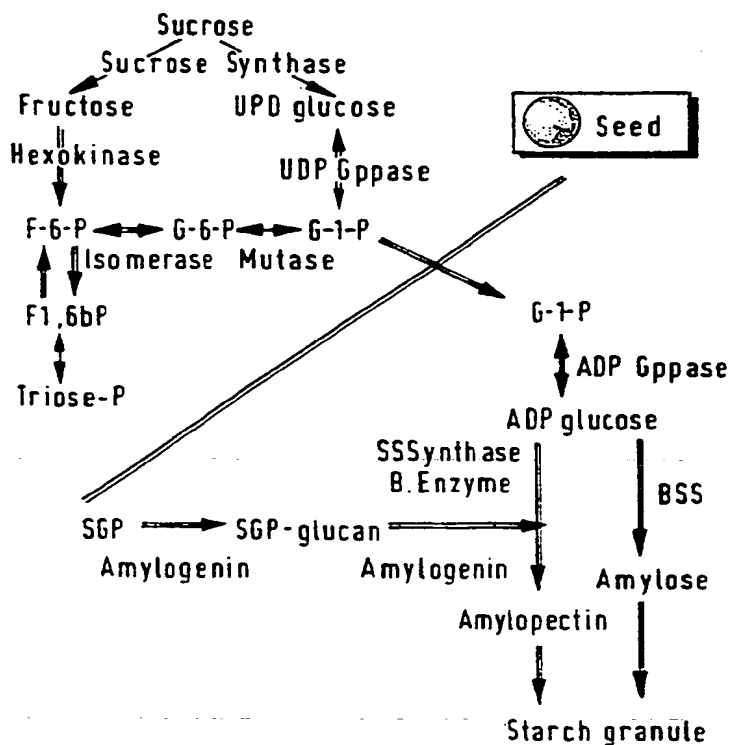| (51) International Patent Classification ^{5} : C12N 15/82, 15/54, A01H 5/00	A2	(11) International Publication Number: **WO 94/04693**
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(57) Abstract

Plants with an altered starch synthesising ability are produced by incorporating into the genome of the plant at least one donor gene encoding a starch primer. The starch primer is an enzyme capable of inititiating starch synthesis, such as amylogenin and/or glycogenin and/or protoglycogenin. DNA constructs encoding a starch primer are provided.

Enzymes of starch synthesis

NOVEL PLANTS AND PROCESSES FOR OBTAINING THEM

This invention relates to novel plants having an altered ability to synthesise starch, and to processes for obtaining such novel plants.

Starch is an important end-product of carbon fixation during photosynthesis in leaves and is an important storage product in seeds and fruits. Starch comprises up to 75% of the grain dry weight in cereals. In economic terms, the starch produced by the edible portions of three grain crops, wheat, rice and maize, provide approximately two-thirds of the world's food calculated as calories.

Starch is synthesised in the plastid compartment (the chloroplast in photosynthetic cells or the amyloplast in non-photosynthetic cells). The biochemical pathway of starch biosynthesis in leaves has been well-characterised and is shown in Figure 1. The abbreviations used are: G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; P_i, orthophosphate; PP_i, inorganic pyrophosphate; ADPG/ADPglucose, adenosine diphosphate glucose; ATP, adenosine triphosphate; UDPG/UDPglucose, uridine diphosphate glucose. The reactions are catalysed by the following enzymes: 1) phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase, 2) triose-phosphate isomerase, 3) aldolase, 4) fructose-1,6-bisphosphatase, 5) hexose phosphate isomerase, 6) phosphoglucomutase, 7) ADP-glucose pyrophosphorylase, 8) starch synthase, 9) UDP-glucose pyrophosphorylase, 10) sucrose phosphate synthase, 11) sucrose phosphatase, 12)

orthophosphate/ triose phosphate translocator, 13)
inorganic pyrophosphatase.

Knowledge of the pathway in storage tissues
was greatly improved following the development of a
method for the isolation of intact amyloplasts from
wheat endosperm (apRees, 1990) and our recent
research with ^{13}C labelling studies in wheat and
maize (Keeling et al, 1988, Plant Physiology, 87:
311-319; Keeling, 1990, ed. C.D. Boyer, J.C.
Shannon and R.C. Harrison, pp.63-78, being a
presentation at the 4th Annual Penn State Symposium
in Plant Physiology, May 1989). Figure 2 shows the
metabolic pathway of starch biosynthesis in maize
endosperm. The abbreviations used are the same as
in Figure 1. The reactions are catalysed by the
following enzymes: 1) sucrose synthase, 2)
UDP-glucose pyrophosphorylase, 3) hexokinase, 4)
phosphoglucomutase, 5) hexose-phosphate isomerase,
6) ATP-dependent phosphofructokinase, 7)
PP_i-dependent phosphofructokinase, 8) aldolase, 9)
triose-phosphate isomerase, 10) hexose-phosphate
translocator, 11) ADP-glucose pyrophosphorylase,
12) starch synthase, 13) sucrose phosphate
synthase, 14) sucrose phosphatase.

Despite this considerable progress, the first
priming molecule(s) responsible for initiating
starch synthesis in plants have remained an enigma.

In the animal kingdom, glycogen is deposited
rather than starch. It is now well established
that glycogen synthesis is initiated on a protein
primer which remains covalently attached to the
mature macromolecule. Recent work on glycogen
deposition has identified a new enzyme: glycogenin

or self glucosylating protein (SGP) which acts as a
primer. The protein primer serves as the anchor on
which the oligosaccharide is constructed and on
which glycogen is built (Kennedy et al, 1985,
Membranes and Muscle ed. by MC Berman et al, IRL
Press Oxford/ICSU Press, pp65-84). The linkage
between the oligosaccharide and glycogenin involves
a novel carbohydrate-protein bond, via the hydroxyl
group of tyrosine (Rodriguez and Whelan, 1985,
Biochem Biophys Res Commun, 132:829-836). The
discovery of glycogenin as a covalent component of
the glycogen molecule was followed by the discovery
in muscle and other tissues of a glycogen-free form
that could be revealed by glucosylation from
^{14}C-labelled UDPglucose (Rodriguez et al, 1986,
Proc 18th Miami Winter Symposium, ICSU Short
Reports, 4:96-99). On purification to homogeneity,
the protein proved to be autocatalytic. It
glucosylates itself and in doing so constructs a
maltosaccharide chain on itself that primes
glycogen synthesis by glycogen synthase and
branching enzyme (Lomako et al, 1988, FASEB J,
2:3097-3103). The self-glucosylating protein (SGP)
in muscle is not present as such, but is part of a
larger molecule (proglycogen) (Lomako et al, 1990,
FEBS Lett, 268:8-12; 1991, FEBS Lett, 279:223-228).
The linkage of glycogen to tyrosine in glycogenin
has been confirmed by Smythe et al (1988, EMBO J,
7:2681-2686), as has the autocatalytic nature of
glycogenin (Pitcher et al, 1988, Eur J Biochem,
176:391-395). The glycogenin protein from rabbit
skeletal muscle has been sequenced by Campbell and
Cohen (1989, Eur J Biochem, 185:119-125).

The formation of a glycoprotein primer may be
a universal feature for the synthesis of

polysaccharides. Several proteins have been
identified as potential priming molecules for
carbohydrate synthesis. None of these proteins,
however, has been associated with a polysaccharide
macromolecule. Other than mammalian glycogen, the
presence of a covalently bound protein (primer) in
a mature carbohydrate molecule has yet to be
demonstrated.

According to the present invention there is
provided a method of producing a plant with altered
starch synthesising ability comprising stably
incorporating into the genome of a recipient plant
at least one donor gene encoding a starch primer.

This invention is based on our discovery that
the enzyme reactions catalyzed by glycogenin are
also important in the starch biosynthetic pathway
of plant organs. We have identified a new protein
responsible for initiating starch synthesis in
plants and have named this plant primer enzyme,
amylogenin or self-glucosylating protein (SGP). We
have therefore redefined the metabolic pathway of
starch biosynthesis in plants to incorporate the
starch priming reaction catalyzed by amylogenin.
Figure 3 shows the new pathway of starch synthesis
in maize endosperm. The abbreviations used are:
UDPGppase (UDP-glucose pyrophosphorylase);
ADPGppase (ADP-glucose pyrophosphorylase); BSS
(bound starch synthase); SSSynthase (soluble starch
synthase); BE (branching enzyme).

Starch (amylose and amylopectin) will be
synthesized only where the initiator protein
(primer) is present and in amounts corresponding to
the amount of primer. Amylogenin may determine how

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much starch is laid down and where it is laid down.
It is therefore possible to genetically manipulate
the starch content of plants by altering the level
and distribution of amylogenin. The genetic
manipulation of starch-bearing plants of commercial
importance, particularly cereals such as maize, may
result in new strains that are advantageous either
for direct food uses, or other uses of starch, or
as sources of starch to be used as a chemical
feedstock. For example, the ratio of amylose and
amylopectin in corn starch may be altered which
would create starches of considerable utility
because of the diverse properties endowed on the
different starches as thickening agents, edible
food wrapping, etc. This route to new plant
varieties complements the traditional and empirical
processes of plant breeding.

The basic technology is of potential
application to the manipulation of any storage
polysaccharide and includes the possibility to
suppress polysaccharide synthesis, as well as to
enhance it, thereby altering the carbohydrate
profile of the plant in directions that may be more
favourable for nutritional or industrial uses.

The method according to the invention is
generally applicable to all plants producing or
storing starch. The recipient plant may be: a
cereal, in particular members of the family
Gramineae such as maize (corn), wheat, rice,
sorghum or barley; a fruit-producing species such
as banana, apple, tomato or pear; a root crop such
as cassava, potato, yam or turnip; an oilseed crop
such as rapeseed, canola, sunflower, oil palm,
coconut, linseed or groundnut; a meal crop such as

soya, bean or pea; or any other suitable species.

Each donor gene encodes a starch primer (a protein molecule capable of initiating starch synthesis). The primer may be amylogenin and/or glycogenin, including amylogenin of plant origin and/or glycogenin or protoglycogenin of bacterial origin and/or glycogenin of fungal origin and/or glycogenin of animal origin.

A donor gene may be derived from any suitable source including a plant, a bacterium, a fungus or an animal cell. For example, suitable sources of plant genes include plants of the species <u>Zea</u> <u>mays</u>, <u>Zea</u> <u>diploperennis</u>, <u>Zea</u> <u>luxurians</u>, <u>Zea</u> <u>perennis</u>, <u>Zea</u> <u>tripsacum</u>, <u>Zea</u> <u>parviglumis</u>, <u>Zea</u> <u>mexicana</u> or teosinte.

A donor gene may be incorporated into the recipient plant genome by sexual crossing of the recipient plant and a sexually compatible donor plant. Alternatively, a donor gene may be isolated and then incorporated into the recipient plant genome by genetic transformation.

The donor gene may be derived from cDNA or genomic DNA (gDNA) encoding a starch primer, or it may be synthesised <u>ab</u> <u>initio</u> using standard techniques. Typically the donor gene encodes the complete primer in the sense orientation so that the transcription product (mRNA) can be translated into the active starch primer enzyme. Alternatively, the donor gene may encode a portion of the starch primer in the sense orientation or may encode some or all of the primer in the antisense orientation so that the transcribed mRNA

inhibits expression of the primer enzyme. It is possible to insert more than one copy of the donor gene into the recipient genome. At least one of the donor genes may encode a modified allelic form of the starch primer enzyme having altered characteristics (such as increased or decreased activity, or differing interactions with other enzymes).

It can be seen that the recipient plant's starch synthesising ability may be altered in various ways depending on the particular donor gene(s) which are incorporated. The total amount of starch produced (starch yield) may be increased or decreased; the rate of starch synthesis may be increased or decreased; the starch produced may have an altered structure; the ratio of amylose to amylopectin may be altered; the temperature optimum of starch synthesis may be increased or decreased; the capacity to produce starch at an elevated or lowered temperature may be improved. These effects on starch synthesis are due directly or indirectly to altered activity of the starch primer enzyme(s). For example, interaction of the starch primer enzyme with other starch synthesising enzymes (such as soluble starch synthase or branching enzyme) may affect the degree, rate and/or type of starch production.

To increase the plant's capacity to produce starch, possible methods include:
(1) Increasing the amount of the starch primer enzyme(s) in a recipient plant by the insertion of one or more additional copies of a normal donor gene encoding the primer(s).
    The source of these additional copies may be

the recipient line itself as the technique would simply increase the amount of enzyme available in the grain rather than change of the properties of the enzyme(s). The gene promoters and other regulatory sequences may also be modified using known techniques to achieve increased amounts of the enzyme in the recipient plant: increased gene expression may be elicited by introducing multiple copies of enhancer sequences into the 5'-untranscribed region of the donor gene.

(2) Insertion of one or more donor genes encoding a starch primer enzyme with enhanced activity.

Achieving this requires the identification of a source of the enhanced activity enzyme gene. Such a source need not be a sexually compatible species as donor genes may be introduced using genetic manipulation techniques. Genetic material may thus be obtained from any source having a primer enzyme with the desired activity characteristics. Exogenous genetic material may alternatively be engineered to give the desired characteristics to the enzymes. Techniques are known in so-called "protein engineering" which can alter the characteristics of an enzyme.

The invention further provides a plant having at least one donor gene encoding a starch primer stably incorporated into the plant's genome such that the plant's ability to synthesise starch is altered. The starch primer may be amylogenin and/or glycogenin.

Presence of the donor gene(s) encoding a starch primer alters the natural process of starch production in the plant. It may alter the rate of starch production, the starch yield, the structure

of the starch produced and so on. For example, such transgenic plants may have an increased capacity to produce starch and so are capable of producing higher yields and/or are capable of producing starch at a faster rate. The alteration may be an indirect effect: for example, presence of the donor gene(s) may change the natural expression ratios of the enzymes in the starch biosynthetic pathway leading to altered starch synthesis, including a different amount of starch or a different branching pattern in the starch.

The invention also provides DNA encoding amylogenin and/or glycogenin. Suitable cDNA or gDNA clones may be isolated from cDNA or gDNA libraries using standard cloning techniques. These cDNA or gDNA clones may then be used as probes for equivalent genes in other species, particularly starch-producing organisms. For example, the enzyme amylogenin may be isolated by subjecting a crude plant extract containing the enzyme (eg maize endosperm) to successive purification by gel chromatography. Antibodies to the substantially pure amylogenin protein may be produced. Such antibodies may be used to screen cDNA libraries for the identification of amylogenin clones.

A cDNA clone encoding amylogenin was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA under the terms of the Budapest Treaty on 19 August 1993 under the accession number ATCC 69389.

The amylogenin and/or glycogenin DNA clones may be used to alter starch production and starch quality in plants by the method described above.

DNA encoding amylogenin and/or glycogenin may be
transferred into plants by sexual crossing or by
transformation. For example, a transgenic maize
plant containing an amylogenin DNA clone may have
increased starch yield. In another example, a
transgenic maize plant containing anti-sense DNA
clones may have decreased starch yield but
increased sugar content.

The DNA clones may also be used to express the
enzyme amylogenin and/or glycogenin in a biological
organism such as E coli.

The present invention will now be described,
by way of illustration, by the following examples
and with reference to the accompanying drawings of
which:
    Figure 1 shows the biochemical pathway of
starch biosynthesis in leaves.
    Figure 2 shows the biochemical pathway of
starch biosynthesis in storage tissues.
    Figure 3 shows the new biochemical pathway of
starch biosynthesis in storage tissues.
    Figure 4 shows the proposed mechanism of
glycogen synthesis.
    Figure 5 shows a model for the mechanism of
glycogen biogenesis in muscle.
    Figure 6 shows the separation of sweet corn
amylogenin (SGP) on DEAE-Sepharose.
    Figure 7 shows the fractionation of sweet corn
amylogenin (SGP) on Sepharose Cl-6B.
    Figure 8 shows the separation of prelabelled
amylogenin (SGP) on Sepharose Cl-6B.
    Figure 9 shows the refractionation of the
combined amylogenin (SGP)-containing fractions.

1.   BACKGROUND TO THE INVENTION

1.1   A model for the biogenesis of glycogen in animal muscle

The proposed mechanism of glycogen synthesis, wherein glucose residues from UDPglucose are fisrt added to the protein primer by glycogen initiator synthase to form oligosaccharide chains that will serve as primers for glycogen synthase and branching enzyme (Krisman and Barengo, 1975), is illustrated in Figure 4.

Present knowledge now allows the construction of a model for the mechanism of glycogen biogenesis in muscle which is illustrated schematically in Figure 5. Following the biosynthesis of the priming protein, namely glycogenin, the first step in the biogenesis of glycogen involves the covalent attachment of glucose to Tyr 194 in glycogenin by a protein tyrosine glucosyltransferase that has yet to be identified. The glucosyl-glycogenin so formed is autocatalytic and extends the covalently attached glucose into a maltosaccharide chain by adding another seven glucose units to itself. Maltooctaosyl-glycogenin then primes the synthesis of proglycogen (Mr 400kDa) by proglycogen synthase and branching enzyme. Macromolecular glycogen (Mr 10,000kDa) is synthesized from proglycogen by the classical glycogen synthase with the assistance of a branching enzyme which may or may not be the same as that utilized in the synthesis of proglycogen.

The mechanism of the initial glucosylation of Tyr 194 in glycogenin remains to be elucidated. While a distinct protein tyrosine

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glucosyltransferase enzyme is envisioned in catalyzing this critical first step, it is possible that glycogenin itself fulfills this role and catalyzes the glucosylation of Tyr 194 before proceeding to elongate the initial glucose unit into a maltosaccharide chain. While the nascent glucose may be extended into a chain of eight glucose residues, proglycogen synthase may act before the chain has reached its full length. In other words, the maltooctaose unit that is constructed on glycogenin after undergoing complete self-glucosylation may not be a prerequisite for the priming of proglycogen synthesis and chains of smaller lengths also may serve as the glucan primer.

The identity of proglycogen synthase also remains to be determined. Whether it is a distinct enzyme or a form of glycogen synthase with a specificity for proglycogen has to be elucidated. If the latter is the case, then the manner in which it differs from classical glycogen synthase will be interesting to examine and may provide additional clues to understanding the intricate regulation of glycogen metabolism.

1.2   A universal mechanism for the biogenesis of polysaccharides

The protein primer model for the biogenesis of mammalian glycogen may extend to starch synthesis and perhaps to polysaccharide synthesis in general. Simple tests using an antibody to glycogenin have indicated that glycogenin-like material may be present in vertebrates, invertebrates, plants, yeast, fungi and bacteria containing glycogen and

starch (Gieowar-Singh et al, 1988, FASEB J, 2(1518):A557). The presence of glycogenin-like proteins in a wide variety of lifeforms suggest that similar protein-priming mechanisms for polysaccharide synthesis may be occurring in these species.

The priming mechanism for starch biosynthesis had hitherto not been elucidated and the nature of the priming molecule in vivo remained unknown. However, the present evidence points to a protein primer.

Similar to Krisman's first observation of protein-bound glycogen synthesis with a high speed pellet from a rat liver homogenate, the synthesis of alpha, 1-4 glucan covalently linked to protein was achieved with a potato tuber particulate preparation. A two step mechanism for the formation of the protein-bound alpha, 1-4 glucan in the potato tuber was subsequently proposed. The first step involved the transfer of a glucosyl residue specifically from UDPglucose to an endogenous protein thereby forming a peptidyl-glucose bond. The enzyme that catalyzed the glucosylation was referred to as UDPglucose : protein transglucosylase 1 and it functioned at micromolar concentrations of UDPglucose. In the second step, the glucosylated protein then serves as a primer for the formation of protein-bound alpha, 1-4 glucosidic chains by accepting glucose units transferred from UDPglucose, ADPglucose or glucose 1-phosphate. It was proposed that the chain elongation, which occured in the presence of millimolar concentrations of the glucose donor, was catalyzed by the endogenous starch synthase or

phosphorylase in the preparation.

The protein undergoing glucosylation in the
first step had a molecular weight of 38kDa and the
added glucose was resistant to hydrolysis by alpha-
and beta-amylase. Glucoamylase, however, was
capable of hydrolysing the protein-bound glucose
which was subsequently identified by paper
chromatography. Furthermore, the added glucose
underwent beta-elimination upon alkaline treatment
in the presence of sodium borohydride and was
thereby converted to sorbitol as determined by
paper electrophoresis. The conversion of the added
glucose to sorbitol implied that the glucosyl
moiety was transfered directly to an aminoacyl
residue on the acceptor protein and furthermore
that the glucan synthesized during the first step
consisted of a single glucose unit. In addition,
the susceptibility to mild alkaline hydrolysis
suggested that the glucose was linked to a serine
or threonine residue by an O-glycosidic bond. This
was subsequently confirmed by reduction of the
beta-elimination products with tritiated sodium
borohydride. Tritiated alanine and tritiated 2-
aminobutyric acid, expected by ^{3}H-sodium
borohydride reduction of the 2-amino acrylic acid
and 2-amino crotonic acid generated by beta-
elimination of carbohydrates attached to serine and
threonine residues, were both obtained. Therefore,
both serine and threonine residues seemed to be
involved in the linkage to glucose.

The ability of the glucosylated protein formed
during the first step to serve as a primer for the
synthesis of protein-bound alpha, 1-4 glucan was
shown by conversion of the former into higher

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molecular weight material as the attached glucose
underwent chain elongation. This was achieved by
first allowing the acceptor protein to undergo
glucosylation with micromolar concentrations of
UDP-[^{14}C]glucose during the first step. Unlabelled
UDPglucose, ADPglucose or glucose 1-phosphate was
then added to the incubation mixture in millimolar
concentrations as substrates for the endogenous
chain-lengthening glucosyltransferases in the
potato tuber preparation.

The increase in the molecular weight of the
radioglucosylated protein formed during the first
step, was observed by analysis of the final
products on urea-SDS-PAGE (sodium dodecyl sulphate-
polyacrylamide gel electrophoresis) and
fluorography. In addition to the 38kDa
radiolabelled band representing the product formed
during the first step a labelled band at 50kDa was
observed on a 12.5% acrylamide gel for the
incubation in which unlabelled UDPglucose was added
during the second step. When a 10% acrylamide gel
was used, the mobility of the band decreased and
now migrated to the 70kDa position. Similar gel
patterns were obtained with the incubations in
which unlabelled ADPglucose and glucose 1-phosphate
were used as substrates for chain elongation.

Fractionation of the solubilized potato tuber
preparation on DEAE-cellulose separated the
endogenous acceptor protein, which copurified with
the enzyme catalyzing its glucosylation during the
first step, from the glucosyltransferases that
participated in the chain-elongation process in the
second step. Furthermore, phosphorylase emerged
later than the acceptor protein as two overlapping

peaks, each of which also contained starch synthase activity. The glucosyltransferase activities that catalyzed the chain extension of the 38kDa glucosylated acceptor protein had copurified with the first phosphorylase peak which was used to reconstitute the synthesis of protein-bound alpha, 1-4 glucan. Chain extension was demonstrated by the increase in the molecular weight of the 38kDa radioglucosylated acceptor protein on SDS-PAGE and fluorography.

The chain lengthening enzymes contained in the first phosphorylase peak utilized UDPglucose, ADPglucose and glucose 1-phosphate as glucosyl donors. Major radiolabelled bands at 43kDa and 50kDa were observed on 12.5% acrylamide gels when UDPglucose and ADPglucose were used as chain-lengthening substrates. When glucose 1-phosphate was used, only the 50kDa labelled band was obtained with the 12.5% gel, which migrated as a 70kDa species on a 10% gel. The 70kDa species, following excision and elution from the gel, was partially converted to a species of 38kDa upon treatment with beta amylase. This supported the contention that the 70kDa species, or at least part of it, originated from the 38kDa glucosylated acceptor protein and was synthesized as the latter underwent chain elongation by the addition of alpha, 1-4 glucose moieties.

The enzymic activity that catalyzes the initial glucosylation of the acceptor protein was recently purified. During purification, the glucosyltransferase and the acceptor protein copurified to homogeneity demonstrating that both were one and the same protein. In other words, the

glucosyltransferase is an autocatalytic enzyme that undergoes self-glucosylation to form the protein primer required for alpha, 1-4 glucan synthesis in the potato tuber.

A similar autocatalytic glycosyltransferase with a molecular weight of 40kDa has been purified from Daucus carota L (carrot) cells. The enzyme functions in micromolar concentrations of UDPglucose from which it tranfers glucosyl moieties onto itself. Treatment of the radioglucosylated product with weak alkali (0.1N NaOH, 24°C, 24hr) and strong alkali (1N NaOH/1M NaBH$_4$, 100°C, 10hr) partially separated the carbohydrate from the protein. Methylation of the oligosaccharides so released produced 2,3,4,6 tetra-O-methyl-glucitol-acetate as the only labelled carbohydrate indicating that autoglucosylation added only one glucose residue to the protein. In addition to UDPglucose, the enzyme also utilized UDPgalactose as a substrate undergoing autogalactosylation. While this carrot enzyme is similar to the autocatalytic protein that appears to prime alpha, 1-4 glucan synthesis in the potato tuber it has yet to be implicated in polysaccharide biogenesis.

The biosynthesis of beta-glucans also appears to proceed through a glycoprotein intermediate. The formation of protein-linked oligosaccharides that seemed to be involved in the synthesis of beta-glucans was detected in bacteria, fungi, algae and higher plants. However, the evidence that a glycoprotein primed beta-glucan synthesis in these studies was circumstantial and a covalent linkage between a beta-glucan chain and a protein was never demonstrated.

In the case of Chlorophyta Prototheca Zopfii
(algae) the evidence is more convincing that beta-
glucan synthesis is initiated on a protein primer.
An intermediate in the synthesis of beta-1,4/1,3-
glucan in this alga displayed properties that were
consistent with a glycoprotein nature. In addition
to being insoluble in trichloroacetic acid, this
intermediate was not dialyzable, strongly absorbed
at 280nm and gave a positive reaction with
phenol/sulphuric acid, Lowry and Buiret reagents.
It contained glucose incorporated from UDPglucose
and was shown to be a precursor of alkali-insoluble
polysaccharides. This was demonstrated by the
synthesis of radiolabelled alkali-insoluble
polysaccharides when the radioglucosylated
intermediate was incubated with unlabelled
UDPglucose and the endogenous enzymes in a fresh
membrane preparation of the alga. The
polysaccharides so synthesized however seemed to be
free of protein indicating that at some point in
the elongation process the priming protein was
discarded.

This intermediate in beta-glucan synthesis was
subsequently isolated and characterized. Membrane
preparations from Prototheca Zopfii cells pulsed
with [^{14}C] proline were incubated with
UDP[^{3}H]glucose. The intermediate so formed was
double-labelled containing both ^{3}H-and ^{14}C-
radioactivity. It was susceptible to partial
hydrolysis by cellulase and beta-glucosidase and
underwent partial degradation with protease. On gel
filtration chromatography, it behaved as a 28000-
30000 Da molecule and migrated as a species of the
same molecular weight on SDS-polyacrylamide gel

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electrophoresis. These characteristics firmly
established that the intermediate was a
glycoprotein with beta-glycosidic bonds in the
carbohydrate moiety.

The double-labelled glycoprotein intermediate
was resistant to beta-elimination (0.15M NaOH, 1M
NaBH$_4$, 100°C, 5hr) indicating that the carbohydrate
moiety was not linked by an O-glycosidic bond to
serine/threonine or by an N-glycosidic bond to
asparagine. Treatment with saturated barium
hydroxide (0.2M Ba(OH)$_2$, 100°C, 6hr) however,
liberated a double-labelled aminoacyl-
oligosaccharide from the glycoprotein. This was
consistent with an O-glycosidic linkage to
hydroxyproline or hydroxylysine whereby cleavage of
the peptide bonds to the glycosylated amino acid
occurs. The association of ^{14}C-label with the
liberated oligosaccharide moiety suggested that the
carbohydrate was linked to hydroxyproline derived
from the radioactive proline that was fed to the
cells.

Cellulase digestion of the double-labelled
product obtained by barium hydroxide treatment
produced a smaller double-labelled aminoacyl
oligosaccharide which upon acid hydrolysis and
subsequent reduction with tritiated sodium
borohydride yielded [^{14}C] hydroxyproline and [^{3}H]
sorbitol. The formation of sorbitol as the only
alditol following reduction indicated that glucose
was present at the reducing end of the
oligosaccharide chain. This suggested that the
carbohydrate-protein linkage in the glycoprotein
intermediate was an O-glycosidic one between
hydroxyproline and glucose.

2. PURIFICATION OF AMYLOGENIN PROTEIN FROM MAIZE ENDOSPERM

The source of the amylogenin enzyme (or self-glucosylating protein, SGP) was a US inbred line (B73) of *Zea* *mays*.

2.1 Preparation of corn extracts

The kernels were cut from the cobs directly into a food grinder which functioned by continuously extruding the ground material. The ground kernels were collected and expressed through cheese cloth into a beaker surrounded by ice and containing a concentrated extracting solution which, after dilution by the corn juice, gave a final concentration of 50mM Tris-HCL pH 7.4, 5mM EDTA (ethylene diamine tetra acetate), 0.1mM EGTA, 14mM mercaptoethanol, 0.1mM PMSF (phenylmethanesulphonyl fluoride), 1mM benzamidine and 0.1mg/liter of both leupeptin and pepstatin. Following this, the mixture was centrifuged at 3000 x g for 15 minutes at 4°C and the supernatant retained as the cell-free corn extract.

The amount of concentrated extracting solution needed for each preparation was estimated at the outset. The exact volume of the extract could not be predetermined because of variability in the size of the cobs. Therefore, an average volume of 70ml of extract/cob was used for estimating the amount of concentrated extracting solution required. After the final volume of the mixture was measured, the quantity of extracting solution was adjusted to give the desired concentration. The use of a concentrated extracting solution facilitated in

keeping the volume of the extract to a minimum, which from a typical preparation with 15 cobs of corn, ranged between 1100-1300 milliliters.

2.2 Detection and quantitation of Self-Glucosylating Protein (SGP)

SGP was assayed by incubating a sample of the enzyme with 2uM UDP-[^{14}C]glucose, 5mM MnCl$_2$ and 50mM Tris-HCL buffer, pH 7.4 in a 100ul reaction mixture at room temperature for 30 minutes (the reaction mixture was made up by adding, in the following order, 10ul each of 10X solutions of Tris-HCL pH 7.4 buffer, MnCl$_2$ and UDPglucose to a 70ul solution of the enzyme). The reaction was stopped by the addition of 1ml of 10% TCA (trichloroacetic acid). After standing for 10 minutes the sample was passed through a nitrocellulose filter, type HA 0.45uM, washed with about 3ml 10% TCA and approximately 10ml of water and the filter dried under an infrared lamp. Following this, the filter was placed in 4ml of scintillation liquid (Ecolume) and measured for ^{14}C radioactivity in a Searle Delta 300 6890 liquid scintillation counter.

The radioactivity measured on the filter represents ^{14}C-glucose that was incorporated into TCA-precipitable material (assuming no conversion to the glucose molecule into any other compound). In the case of SGP, which functions by transfering a single glucose residue from UDPglucose onto itself, the assay provides a means of quantitating 'naked' SGP. Endogenously glucosylated SGP would not be detectable and could not be quantitated by this method.

In corn, as in many other plant species possessing SGP-like proteins, the assay also detects, in a crude extract, the incorporation of radioactivity into a TCA-precipitable, lipid-like substance. This lipid-like substance is soluble in methanol, methanol/chloroform and ethanol. In a crude extract of corn, this lipid-like substance represents about 80-90% of the TCA-precipitable, radiolabelled material measured by the assay. As SGP is purified away from this substance, however, the assay provides an accurate measurement of SGP activity.

In a modification of the procedure and in an attempt to measure SGP activity in crude extracts, 1ml of 95% ethanol was used in place of 1ml 10% TCA to precipitate protein. After vortexing well to dissolve the lipid-like substance and standing on ice for 30 min to allow protein precipitation, the sample was centrifuged at 7000 rpm for 15 min with a bench top centrifuge. The protein pellet was resuspended in 10% TCA and processed as in the original assay. This provided a way of separating the two activities and allowing SGP activity to be measured in crude extracts.

In addition to scintillation counting, SGP was detected by SDS-PAGE, Western blot analysis and radioautography/fluorography. These procedures, however, were used only for qualitative analyses.

SDS-PAGE was performed with a Mini-Protean 11 electrophoresis system (BioRad) on 10% acrylamide gels according to Laemmli [108]. Samples were electrophoresed in electrode buffer, pH 8.3 (25mM Tris, 192mM glycine, 0.001% SDS) with a 25mA

current for approximately 1 hour. Protein bands
were visualized by staining gels with either
Coomassie blue R-250 or silver (BioRad silver stain
kit). Following this, the stained gels were soaked
in Amplify for 15 min [109] and dried onto Whatman
3MM paper in a slab dryer (BioRad) at 60°C.

Western blotting was performed in a Mini
trans-blot electrophorectic transfer cell (BioRad).
Following SDS-PAGE, proteins from unstained gels
were transfered onto nitrocellulose paper in 25mM
Tris, 192mM glycine, 20% methanol (v/v) pH 8.3
buffer. The transfer was carried out by applying a
150mA current for 1 hr. After transfer, the
nitrocellulose blot was blocked in 5% Carnation
lowfat milk dissolved in Tris-buffered saline (20mM
Tris-HCL, 0.5M NaCl, pH 7.4). After blocking, the
blot was washed 3 times with Tris-buffered saline
(TBS) and then incubated overnight in TBS
containing 1% gelatin with goat anti-glycogenin IgG
(1:1000 dilution of original stock). Next, the blot
was washed 3 times with TBS and then incubated with
alkaline phosphatase-conjugated rabbit anti-goat
IgG. The antigen-bound protein bands were detected
by color development with solutions of BCIP (5-
bromo-4-chloro-3indolyl phosphate) and NBT (p-
nitroblue tetrazolium chloride) as instructed by
the supplier.

Radioautography was performed on dried gels,
immunoblots and thin layer silica plates containing
radiolabelled materials by exposure to Kodak X-OMAT
XAR-5 films at -70°C. (Fluorography was also
performed on dried gels in the same manner). The
films were developed with an automated Kodak X-OMAT
film processor.

2.3   Purification of Self-Glucosylating Protein from Sweet Corn

The following steps were carried out, at 4°C, to obtain homogeneous SGP from sweet corn.

Step 1- Acid precipitation

After preparing a cell-free extract from 15 cobs of sweet corn, the pH was adjusted from 7.4 to 5.0 with 1M ammonium acetate pH 5.0 buffer. Precipitation was allowed to occur for 1 hr. The supernatant, which contained the protein glucosylating activity, was collected by centrifugation at 4000 x g for 15 min. Most of the lipid-glucosylating activity remained in the pellet.

Step 2- Fractionation by salt precipitation

Solid ammonium sulphate was added to the pH 5.0 supernatant to give a 50% saturated solution. Protein was allowed to precipitate overnight and collected by centrifugation at 10,000 x g. The protein pellet was resuspended in 50mM Tris-HCL pH 7.4 buffer containing 2mM CHAPS (TC) and dialyzed against the same buffer. Following dialysis, the solution was centrifuged to remove the considerable amount of undissolved material.

Step 3- Fractionation by ion exchange chromatography

The dialyzed solution was applied to a DEAE-Sepharose column (6 x 15cm) that was equilibrated with TC. After passing equilibrating buffer through the column to remove unbound material, the protein was desorbed with an increasing concentration gradient of NaCl, from 0 to 1M, formed from 500ml

of TC and 500ml of TC containing 1M NaCl (Figure
6). Fractions of 11ml were collected and assayed
for protein-glucosylating activity. The fractions
possessing the highest activity were pooled and
concentrated to approximately 25ml by
centrifugation using centriprep-10 concentrators
(Amicon).

Step 4- Fractionation by gel filtration
chromatography
    The concentrated material was applied, in two
equal portions during separate runs, to a Sepharose
Cl 6B column (3 x 85cm) that was equilibrated with
TC and eluted with the same buffer (Figure 7). The
protein-glucosylating activity was found in a
single, but broad, peak that began at the void
colume. The material that emerged at the front,
represented a single protein species on a silver-
stained gel after SDS-PAGE and possessed both the
glucosylating activity and the glucosylation site.
These early fractions containing homogenous protein
represented about 10% of the total glucosylating
activity applied to the column. The later fractions
contained more activity but were increasingly less
pure with each additional fraction.

2.4 Purification of Prelabelled SGP from Sweet
Corn
    The purification of the self-glucosylating
protein from sweet corn was accomplished by
successive precipitation with acid and salt
followed by column chromatography on DEAE-Sepharose
and Sepharose Cl-6B.

TABLE 1

Purification of sweet corn amylogenin (SGP)

Purification Step	Specific Activity (x103 cpm/ mg protein)	Overall Yield %	Fold Purific'n
crude extract	5	100	1.0
pH 5.0 supernatant	7	53	1.4
50% Ammonium Sulphate ppt	8	42	1.7
DEAE-Sepharose	82	28	16.4
Sepharose	10700	3	2140

A 3% yield (Table 1) representing approximately 2 nanomoles or 80ug of the protein was attained from the preparation with 15 cobs of corn. The homogenous SGP so obtained, however, was difficult to process and required additional purification. It appeared that the protein was complexed to lipid. Additional purification to separate the protein away from the contaminating lipid would further reduce the already low yield of the enzyme and therefore, in order to obtain a sufficient quantity of the protein for amino acid and sequence analyses, it was deemed necessary to purify a larger amount of the SGP.

Two approaches were envisioned to obtain an adequate amount of SGP. One was to purify several extracts individually and combine the homogenous protein from each preparation. The other was to purify, in addition to the homogenous protein at the front of Sepharose Cl-6B, the impure SGP that

elutes later. The first approach was attempted and approximately 4 nanomoles of electrophoretically homogenous SGP was obtained from two preparations with 15 cobs of corn each. In a series of experiments to find an appropriate method to purify the protein from the lipid, most of the material was lost.

In the next attempt to isolate sufficient protein, it was decided to try the second approach by purifying the SGP that elutes later on Sepharose Cl-6B together with the portion that emerges at the front as the single protein species. It was not necessary to purify the protein in an active state since the objective was to obtain the amino acid composition and amino acid sequence information on the protein. Therefore, the strategy was to partially purify the SGP, then prelabel the enzyme by allowing it to undergo self-glucosylation and finally isolate the prelabelled protein. The prelabelling of the SGP prevented losses from inactivation of the enzyme during subsequent purification and allowed procedures that involve denaturing conditions to be employed.

The following steps were performed to isolate the self-glucosylating protein from sweet corn in a prelabelled form and was accomplished in two phases.

Phase 1 - Partial purification of the self-glucosylating protein and subsequent prelabelling by autoglucosylation:

     Step 1 - Acid precipitation;

     Step 2 - Salt precipitatio;n

     Step 3 - Fractionation by ion exchange

chromatography.
Steps 1-3 are exactly the first three steps in the
protocol for purifying active SGP (section 2.3) and
were carried out in the same manner. Following the
fractionation on DEAE-Sepharose and concentration
of the SGP containing fractions, the enzyme was
allowed to undergo self-glucosylation, 25% with
UDP-[^{14}C]glucose and the remaining 75% with
unlabelled UDPglucose. The prelabelled protein was
then purified further.

Phase 2 - Purification of the prelabelled SGP:
    Step 4 - Fractionation by gel filtration
chromatography.
    At the end of the prelabelling reaction, the
incubation mixture was applied to a Sepharose Cl-6B
column (3 x 85cm) equilibrated with 50mM Tris-HCL
pH 7.4 buffer containing 0.02% triton and eluted
with the same buffer (Figure 8). Fractions were
collected in a volume of 5.5ml each. The
prelabelled SGP was detected by measuring 100ul
aliquots from alternating fractions for ^{14}C-
radioactivity. The prelabelled protein emerged in a
broad peak similar to that observed for the native
unglucosylated enzyme. A peak of ^{14}C-radioactivity
detected at the total volume of the column
represented unreacted radiolabelled UDPglucose from
the prelabelling reaction.
    The purity of the prelabelled SGP was examined
by SDS-PAGE in which the protein bands were
visualized by silver staining. Fifty microliter
(50ul) aliquots from every third fraction
containing prelabelled SGP were analyzed in this
manner. The fractions containing the prelabelled
protein were then separated into two sets based on
their protein content depicted by the silver

stained polyacrylamide gel. The earlier fractions
(43-51) containing purer SGP were pooled together
and the later fractions (52-60) possessing SGP in a
less pure state were combined into a second pool.
The pools of prelabelled SGP were concentrated by
centrifugation using centriprep-10 concentrators
(Amicon).

Step 5 - Fractionation by Reverse-Phase HPLC.

Following the separation on Sepharose Cl-6B,
the prelabelled SGP was purified next by reverse-
phase HPLC on a Perkin ELmer HPLC system using a
Vydac C_4 column (8 x 40mm). The sample was
fractionated on the column in several portions.
During each separation 450ul aliquots containing
0.26mg of total protein were applied to the column.
This was done to obtain the best resolution of the
SGP containing peak. It was observed, during the
purification of prelabelled SGP from B73 commercial
dent corn, that a larger amount of protein on this
column resulted in a lower resolution. The protein
was desorbed from the column with linear gradients
of acetonitrile generated by using 0.1% TFA
(trifluoroacetic acid) in water (solution A) and
0.09% TFA in water containing 84% acetonitrile
(solution B) at a flow rate of 1ml/min. The first
two separations were performed successfully with a
linear gradient of 0-100% solution B applied over a
period of 50 minutes followed by an additional 10
minutes with 100% solution B. In an effort to
reduce the time for each run, further separations
were carried out with a gradient composed of the
following linear segments : 0-30 minutes, 0-70%
solution B; 30-31 minutes, 70%-100% solution B; 31-
41 minutes, 100% solution B. The altered gradient
worked with the same success. Protein was detected
by monitoring the absorbance of the effluent at

215nm and 295nm simultaneously. Peaks containing protein were collected manually and the prelabelled SGP emerged from the column in a single peak.

The fractions containing prelabelled SGP from each run were pooled together. The combined fractions had a volume of 10.7ml and was concentrated to approximately half its volume on a speed-vac. The concentrated sample was then diluted to its original volume with water thereby reducing its acetonitrile concentration by approximately half. The sample was then refractionated on the C_4 column in two portions (Figure 9). In the first run, 5.7ml of the sample was applied to the column by injecting consecutively six aliquots of 950ul each. The remainder of the sample was fractionated in a second run by injecting five 950ul aliquots onto the column. Protein was eluted in the same manner as before and the fractions containing prelabelled SGP from both runs were combined giving a volume of 3.3ml. Protein determination on the combined fractions by amino acid analysis revealed that 149.2ug of protein was present. An aliquot containing 3ug of protein was analyzed on SDS-PAGE for purity. A single homogenous protein band representing prelabelled SGP was observed.

2.5 Reduction and Alkylation of purified SGP

The sample containing 149.2ug of protein in a volume of 3.3ml, was prepared for reductive carboxymethylation by adding to it 400ul of 5M guanidium-HCL, 0.5M Tris-HCL pH 8.6 buffer. After mixing well the solution was concentrated to 0.4ml on a speed-vac. Reduction and carboxymethylation were performed on the protein in the following manner:

Step 1 - Reduction with DTT (20 umol/mg protein).

Five microliters (5ul) of 1.0M DTT was added to the sample and the reaction tube then flushed with argon. The reaction was allowed to proceed by incubation at 50°C for 2 hr.

Step 2 - Carboxymethylation with iodoacetic acid (60 umol/mg protein).

Next, 15ul of 1M iodoacetic acid was added and after flushing the tube with argon the sample was incubated at room temperature for 30 min in the dark.

Step 3 - Reduction with DTT (30 umol/mg protein).

Seven and one half microliters (7.5ul) of 1.0M DTT was added to the sample and then incubated at 50°C for 2 hrs after flushing the tube with argon.

Step 4 - Carboxymethylation with iodoacetic acid (60 umol/mg protein).

Next, 15ul of 1M iodoacetic acid was added and the reaction allowed to proceed at room temperature for 30 min in the dark under argon.

The reaction was terminated by adding 17.5ul of 1.0M DTT to the incubation mixture. Following this, the protein was dialyzed against 50mM Tris HCL pH 7.6 buffer containing 1mM $CaCl_2$ on a Pierce Microdialysis System 500. The progress of dialysis was monitored by following the decrease in conductivity of the sample. This was achieved by taking 2.5ul aliquots from the sample at regular intervals and, after diluting in 10ml of water, measuring the conductivity. Dialysis was stopped after a 100 fold decrease in conductivity was obtained over 2.5 hr. After dialysis, the volume of the sample had increased to 550ul and its pH was 7.8.

3. AMINO ACID COMPOSITION AND SEQUENCING OF AMYLOGENIN (SGP)

3.1 Amino acid composition of sweet corn SGP

An accurate determination of the amino acid composition of sweet corn SGP was made by calculating the expected yield of each amino acid after 100 hr of hydrolysis. This was done by performing amino acid hydrolyses on samples of the protein at 110°C for 24 hr, 48 hr and 72 hr respectively. From a statistical analysis of the yield for each amino acid at each time interval an extrapolation was made for the expected yield at 100 hr and the amino acid composition calculated therefrom. A comparison between the amino acid compositions of sweet corn SGP and glycogenin (Table 2) indicates a general similarity between the two proteins. Other than serine, histidine, threonine and isoleucine, the proportion of each amino acid in the two proteins are comparatively similar. In particular, the ratio of glycine, arginine, alanine, and valine in each protein differ by less than 10% while that of leucine, phenylalanine and lysine are less than 13% different.

TABLE 2

Comparison between the amino acid composition of glycogenin and amylogenin

Amino Acid	Glycogenin	Amylogenin
ASX	1.00	1.00
GLX	0.93	0.67
SER	0.80	0.40
GLY	0.59	0.63
HIS	0.26	0.14
ARG	0.33	0.35
THR	0.84	0.44
ALA	0.59	0.65
PRO	0.44	0.60
TYR	0.26	0.37
VAL	0.63	0.67
MET	0.13	0.21
ILE	0.28	0.60
LEU	0.94	0.84
PHE	0.49	0.44
LYS	0.50	0.44

3.2   Preparation and isolation of tryptic peptides from purified SGP

The sample of purified prelabelled SGP contained 149.2ug of protein. On previous occasions, it was found that the partially pure protein was resistant to trypsinolysis with 70% proteolysis occurring over a 48 hr incubation period. To make the purified protein more susceptible to proteolysis, reduction and carboxymethylation was performed. The protein was then digested with trypsin in an enzyme/substrate ratio of 1:25 for 15 hr. The tryptic digest was fractionated on a C_{18} column by reverse-phase HPLC and peptide peaks collected separately. An examination of the fractions for ^{14}C-radioactivity revealed the presence of 3 radiolabelled peaks.

Nine of the peptide peaks (labelled P1-P9) including the 3 radioactive peaks were further purified by fractionation on a smaller C_{18} column at pH 6.0. Upon refractionation of peaks P1-P9 the following number of tryptic peptides were obtained from each.

Peak Number	No of tryptic peptide peaks obtained
P 1 (fraction 29+30)	4
P 2 (fraction 33+34)	3
P 3 (fraction 37+38)	5*
P 4 (fraction 42)	2
P 5 (fraction 54)	4
P 6 (fraction 55-57)	5*
P 7 (fraction 59)	3
P 8 (fraction 60+61)	1*
P 9 (fraction 62)	1

* ^{14}C-radioactivity containing peaks

The fractionation of P3 and P6 each gave rise to one radiolabelled tryptic peptide peak. The separation of P8, which had very little radioactivity (12000cpm in total), produced only a single peptide peak which was not radiolabelled. No radioactivity was detected in any of the fractions. It appears that the recovery of radioactivity was too small to be detected.

3.3 Amino Acid Sequence of Tryptic Peptides

An attempt to sequence the N-terminus of sweet corn SGP was made by applying a sample of the purified protein for amino acid sequencing. It was found however that the N-terminus was blocked. Nine of the purified tryptic peptides were chosen for amino acid sequence analyses (labelled T1-T9). The

amino acid sequences of these peptides are
summarized below.

T 1 (P 1) - Y V N A V M T I P K

T 2 (P 3) - E G A N F V X G Y P F S L R *

T 3 (P 4) - Y X X M W A G W T V K

T 4 (P 4) - E G A H T A V S H G L W L N I P D Y D A
            P T Q L V K P K

T 5 (P 5) - L G D A M V T W I E A W D E L N P S T P
            A A A D G K

T 6 (P 6) - L G D A M V T D I E A A D E L N P A G P
            X X X X K

T 7 (P 6) - N L L S P S T P F F F N T L Y D P Y R E
            G A N F V X G Y P F S L R *

T 8 (P 7) - G I F W Q E D I I P F F Q N V T I P K

T 9 (P 9) - N L D F L E M W R P F F Q P Y H L I I V
            Q D G D P T K

            * radioglucosylated tryptic peptides

     The radiolabelled peptide T2 is a proteolytic
fragment of the larger radiolabelled peptide T6. It
is likely therefore that the radiolabelled peptide
in P8 was simply a larger peptide comprising T2 and
T6 formed from incomplete proteolysis. The
emergence of the peptide later than T2 and T6
suggest that it has a larger size. The small
quantity of radioactivity (12000cpm) further
suggest that the peptide may have arisen from
incomplete hydrolysis and more so that
trypsinolysis was performed for only 15 hr. In the
amino acid sequence of T2 and T6 no
phenylthiohydantoin amino acid was detected at
position 7 and 26 respectively and most of the
applied radioactivity remained bound to the glass
fibre disc, indicating that the [^{14}C]-glucosylated
amino acid was not extracted by the sequencer
solvents. This implied that the radiolabelled

glucose was attached to the amino acid at this
position.

3.4 Determination of the amino acid involved in
the protein-carbohydrate linkage

In order to characterize the protein-
carbohydrate linkage in the sweet corn SGP, it was
necessary to determine the amino acid to which the
glucose was attached. This was achieved by
performing amino acid analysis and mass
spectrometry on the radioglucosylated peptide, T2.

Amino acid analysis of T2 revealed that the
peptide had the following empirical composition
$D_{1.0}$ $E_{1.0}$ $S_{1.2}$ $G_{2.8}$ $R_{2.0}$ $A_{1.2}$ P_{nd} $Y_{1.1}$ $V_{1.0}$ $L_{1.5}$
(nd = not determined). The composition of the
peptide from sequence analysis was D_1 E_1 S_1 G_2 R_1
A_1 P_1 Y_1 V_1 L_1 X_1. When the two compositions were
compared, the most striking difference was in the
amount of arginine. Sequence analysis had found
only one arginine residue but amino acid analysis
indicated that two molecular proportions were
present in the peptide. By inference, therefore,
the unknown amino acid, X, at position 7 in the
peptide must be arginine.

The peptide T2 was submitted to ion-spray mass
spectrometry in order to determine its precise
molecular mass. The ion-spray mass spectrum of the
peptide yielded only one prominant signal at m/z
(mass/charge ratio) 888.9 corresponding to a
molecular mass of 1777.8 Da for a doubly protonated
ion. The molecular mass of peptide T2 calculated
from its amino acid composition by assuming an
arginine residue in position 7 is 1776.97. By

inference, therefore the signal must represent
peptide T2 and in combination with the amino acid
analysis data confirms that the amino acid to which
the glucose is attached at position 7 is arginine.
It should be noted that minor signals were observed
in the ion spray mass spectrum. These may represent
contaminants in the sample as well as sodium and
potassium salts of the peptide.

Tandem ion-spray mass spectrometry was also
performed on the peptide. A daughter ion spectrum
obtained by collisional activation in which the
doubly protonated parent ion was bombarded with
argon, showed two signals, a prominant one at m/z
889.0 and a minor one at m/z 807.5. The major
signal represents the doubly protonated parent ion
corresponding to the glucosylated peptide T2. The
minor signal is due to a doubly charged daughter
ion generated by fragmentation of the parent ion.
The mass difference between the parent ion and the
daughter ion is 163 Da which corresponds to a
glucosyl fragment. The presence of only one
daughter ion with a loss of a 163 Da fragment from
the parent ion strongly suggests that only one
glucose residue was attached to the peptide. This
implies, therefore, that during the self-
glucosylation reaction by the sweet corn SGP only
one glucose residue is transferred onto the protein
directly to an arginine residue.

3.5   Trypsinolysis of purified SGP
      Following carboxymethylation of the purified
SGP the protein was digested with trypsin to
generate peptides for subsequent sequencing. For
this purpose, TPCK-treated trypsin modified by

reductive alkylation (Promega) was used. The modified trypsin is extremely resistant to autolytic digestion. The lyophillized TPCK-treated, modified trypsin was dissolved first in 50mM acetic acid to a concentration of 1ug/ul. Fifteen microliters (15ul) of this solution was added to the sample containing 144ug of purified SGP in a volume of 530ul giving a protease : protein ratio of 1 : 25 (w/w). The digest was incubated at 37°C for 15 hr.

3.6   Purification of Tryptic Peptides from SGP by Reverse-Phase HPLC

The peptides generated by trypsinolysis on purified SGP were isolated by reverse-phase HPLC on a Perkin Elmer HPLC system using a Vydac C_{18} column (100 x 4mm). At the end of proteolysis the whole digest was applied to the column. Peptides were eluted with a linear gradient between 0.1% TFA in water (solution A) and 0.09% TFA in water containing 84% acetonitrile (solution B) applied over a period of 100 min at a flow rate of 0.4ml/min. Peptides were detected by monitoring the absorbance of the effluent at 215nm and 295nm simultaneously and collected manually.

Individual peaks were then chosen and purified further by refractionation at pH 6.0 on a smaller C_{18} column (50 x 2mm) with the same HPLC system. Each peak was concentrated to half its volume on a speed-vac and then diluted to its original volume with water. This served to reduce the acetonitrile concentration in each sample by approximately half. The samples were refractionated individually by applying each to the C_{18} column. Peptides were eluted from the column with a linear gradient of

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acetonitrile from 0-84% generated from 10mM
ammonium acetate pH 6.0 and 10mM ammonium acetate
pH 6.0 containing 84% acetonitrile. The gradient
was applied over a period of 60 min with a flow
rate of 0.15ml/min. Peptides were detected as
before and collected manually. After this round of
purification chosen peptides were then subjected to
amino acid sequence analyses.

3.7 Amino Acid and Sequence Analyses

Amino acid analyses was performed on both the
intact prelabelled SGP and tryptic peptides
generated therefrom. This was done by the PTC
derivitization procedure on an automated amino acid
analyzer. Samples were dried on a speed-vac and
then hydrolyzed in 6N HCL at 150°C for 70 min.
Following hydrolysis the samples were dried again
on the speed-vac and then 2 nanomoles of
norleucine added to each as an internal standard.
The samples were then applied to the amino acid
analyzer which quantitates the amino acids by
reverse-phase HPLC against amino acid standards.
The HPLC solutions were 50mM sodium acetate pH 5.6
(buffer A) and 30mM sodium acetate pH 4.6
containing 70% acetonitrile (buffer B).

In order to obtain the most accurate amino
acid composition of the intact SGP, samples were
hydrolyzed in 6N HCL at 110°C for 24 hr, 48 hr and
72 hr respectively. The amino acid composition was
then determined by extrapolation for 100 hr of
hydrolysis.

Amino acid sequence analyses were performed by
the Edman degradation procedure on an Applied
Biosystems 470A gas-phase sequencer equipped with

an on line model 120 phenylthiohydantoin analyzer
using the 03R phenylthiohydantoin standard program.

3.8 Mass Spectrometry and Tandem Mass Spectrometry
of Radioglucosylated Tryptic Peptide from
Prelabelled SGP

Ion-spray mass spectrometry and ion-spray
tandem mass spectrometry were performed on the
radioglucosylated tryptic peptide isolated from
purified prelabelled SGP. The ion-spray mass
spectra and ion-spray tandem mass spectra were
recorded on a Sciex (Toronto) AP1 111 triple-
quadrupole mass spectrometer with 2400-Da mass
range equipped with an ion spray source. The mass
spectrometer was operated under unit-mass
resolution conditions for all determinations. The
ion spray voltage was 5kV.

The samples were dissolved in 1% formic
acid/methanol prepared in a 1 : 1 (vol/vol) ratio
and introduced into the ion spray source at a
constant flow rate of 5ul/min with a microliter
syringe by using a medical infusion pump (Harvard
Apparatus). Argon at a target gas thickness of
approximately 1.2×10^{14} atoms per cm^2 was used as
collision gas for tandem mass spectrometry. The
collision energies ranged from 40 to 60 eV.

4.   <u>NATURE OF THE CARBOHYDRATE COMPONENT OF SWEET
CORN SGP</u>

The self-glucosylating protein in rabbit
muscle, as isolated, contains at least one glucose
unit. Self-glucosylation consists in lengthening
this chain to eight units by the addition of 1,4-
linked alpha-glucose residues. The added glucose

residues are therefore removable by glycogenolytic enzymes. In an attempt to characterize the carbohydrate component of the sweet corn SGP the following studies were performed.

4.1 Acid hydrolysis of prelabelled SGP

The first step was to determine whether the radioactivity incorporated into the SGP was in fact radiolabelled glucose and not a byproduct of a conversion reaction. To examine this, SGP was allowed to undergo radiolabelling and then acid hydrolysis performed on the product. An autoradiograph of the hydrolysate after thin layer chromatography included a radiolabelled glucose standard. Under mild hydrolysis, after boiling in 2M trifluoroacetic acid for 30 minutes, a faint radiolabelled band is seen at the position of glucose. In addition, three slower moving radiolabelled bands of stronger intensity were observed. Under stronger conditions, by boiling for 2 hrs, the same radiolabelled bands are observed. Now, however, the radiolabelled band at the position of glucose was much more intense and the two other bands had a slightly less intensity than before. The appearance of the radiolabelled band at the glucose marker after acid hydrolysis indicates that the radioactivity incorporated into SGP is radiolabelled glucose. Furthermore, the incorporated glucose was not bound by an acid labile bond since strong acid hydrolysis was necessary to release a significant amount of the carbohydrate from the protein. It is unclear what the slower moving radiolabelled bands represent, but perhaps these may be radiolabelled peptide fragments from the protein. The marginally lighter intensity observed for strong acid hydrolysis

supports this hypothesis.

4.2 Digestion of prelabelled SGP with glycogenolytic enzymes

The radiolabelled glucose attached to SGP after undergoing radioglucosylation is not removable by glycogenolytic enzymes, namely alpha-amylase, beta-amylase and glucoamylase. It is removable, however, by beta-glucosidase. When a sample was incubated with beta-glucosidase only 50% of the radiolabelled glucose was removed, and only after 72 hr of digestion. The difficulty in hydrolysing the glucose by beta-glucosidase suggested that there may be steric hindrance to the enzyme. Therefore, it was decided to test the beta-glucosidase on a prelabelled tryptic peptide. When this was done, beta-glucosidase removed all the ^{14}C-labelled glucose from the radiolabelled tryptic glucopeptide isolated from the prelabelled SGP. Alpha-Glucosidase failed to remove any glucose from the same glucopeptide and in the intact prelabelled SGP.

The inability of alpha-amylase, beta-amylase and glucoamylase to remove the added glucose to SGP indicated that multiple alpha-glucose residues were not attached to the protein. The removal of the glucose by beta-glucosidase indicates that it is attached via a beta-linkage. These data, in combination with other analyses, suggest that the glucose is attached in a beta-linkage to an arginine residue on the protein.

4.3 Beta-elimination of radioglucosylated SGP

The alkaline borohydride procedure which beta-eliminates carbohydrate attached to serine or

threonine residues on proteins was attempted on radioglucosylated SGP from sweet corn. In this experiment prelabelled SGP from rabbit heart was used as a control since its carbohydrate is attached to tyrosine. Surprisingly, the glucose attached to both the sweet corn and heart proteins beta-eliminated or appeared to do so. After 42 hr of incubation, 63% of the radiolabel from the sweet corn protein and 89% from the heart protein became TCA-soluble. This observation suggested that solubilization of the radiolabel in TCA occurred not by beta-elimination but perhaps by fractionation of the protein into TCA-soluble peptides. It was concluded, therefore, that the conditions of this procedure were to strong for the two proteins.

A second procedure commonly used for beta-elimination by incubation in 0.5M NaOH for 24 hr at 4°C was performed on the sweet corn SGP. In this case, the glucose attached to the protein failed to beta-eliminate suggesting that the carbohydrate was not attached to a serine or threonine residue. This result is consistent with the finding that the glucose is attached to an arginine residue.

4.4 Acid hydrolysis and Thin Layer Chromatography of Prelabelled SGP

A sample of prelabelled SGP from sweet corn was prepared by allowing the enzyme to undergo self glucosylation with UDP-[^{14}C]glucose. This was accomplished by incubating 250ul of a 16 fold purified sample of the protein with 50mM Tris-HCl pH 7.4 buffer, 5mM $MnCl_2$ and 2uM UDP-[^{14}C]glucose in a 500ul reaction mixture at room temperature for 30 min. The reaction was stopped by extensive

dialysis of the incubation mixture against 20mM
Tris-HCl pH 7.4 buffer containing 2mM CHAPS. Two
aliquots of the prelabelled protein (12000cpm each)
were each precipitated with an equal volume of 20%
TCA and the pellets collected by centrifugation.
The precipitates were washed twice in ether to
remove residual TCA. After drying in air, 1.0ml of
2M trifluoroacetic acid was added to the
precipitates. For mild hydrolysis one sample was
heated for 30 minutes in a boiling water bath. For
strong hydrolysis the other sample was heated for
2 hr instead. Following hydrolysis, the samples
were lyophilized and redissolved in 100ul of water.

After hydrolysis, 10ul of each hydrolysate was
spotted on thin layer silica plates (the plates
were heated to 60°C for about 5 mins to remove
moisture before use). After drying, the plates were
placed in tanks containing a solvent composed of
butanol, water and ethanol in a ratio of 5 : 4 : 5.
After 24 hr the plate was taken out, dried in an
oven and then replaced for a second run. After an
additional 24 hr the plate was taken out dried and
placed for radioautography.

4.5 Digestion of Prelabelled Sweet Corn SGP with
Glycogenolytic Enzymes
       A sample of prelabelled SGP from sweet corn
was prepared. Five hundred microliters (500ul) of
a 16 fold purified sample of the protein was
allowed to self glucosylate with UDP[^{14}C]glucose in
a 1000ul reaction mixture. The prelabelled SGP so
formed was used in the following digestions.
4.5.1 ALPHA-AMYLASE DIGESTION
       Three hundred microliters (300ul) containing
22680cpm of prelabelled SGP was incubated with

100mM sodium acetate pH 7.0 buffer, 10mM $CaCl_2$ and 2U of alpha-amylase in a 400ul reaction mixture at room temperature. Hydrolysis of glucose attached to the SGP was monitored by measuring any loss of TCA precipitable counts. One hundred microliter (100ul) aliquots were taken at 1 hr, 3 hr and 18 hr time intervals and precipitated with 1ml of 10% TCA. The precipitate was filtered on nitrocellulose, washed and measured for ^{14}C radioactivity.

4.5.2 BETA-AMYLASE DIGESTION

Three hundred microliters (300ul) containing 22680cpm of prelabelled SGP was incubated with 100mM sodium acetate pH 5.0 buffer and 0.5mM dithiothreitol with approximately 2U of beta-amylase in a 400ul reaction mixture at room temperature. Hydrolysis of glucose attached to the SGP was monitored in exactly the same manner as described before.

4.5.3 GLUCOAMYLASE DIGESTION

Three hundred microliters (300ul) containing 22680cpm of prelabelled SGP was incubated with 100mM sodium acetate pH 5.0 buffer and 4U of glucoamylase in a 400ul reaction mixture at room temperature. Hydrolysis of glucose attached to the SGP was monitored in exactly the same manner as described before.

4.6 Digestion of Prelabelled Muscle SGP with Glycogenolytic Enzymes

This experiment was performed as a control for the glycosidase digestion of prelabelled sweet corn SGP. A sample of prelabelled SGP from rabbit muscle was prepared in a similar manner to that from sweet corn. Two hundred microliters (200ul) of a 20 fold purified sample of the muscle enzyme was allowed to undergo autoglucosylation in a 400ul reaction

mixture for this purpose. The prelabelled muscle SGP was incubated with glycogenolytic enzymes in the following digestions.

4.6.1 ALPHA-AMYLASE DIGESTION

Fifty microliters (50ul) containing 4907cpm of prelabelled muscle SGP was incubated with 100mM sodium acetate pH 7.0 buffer, 10mM $CaCl_2$ and 2U of alpha-amylase in a 100ul reaction mixture at room temperature. Hydrolysis of glucose attached to muscle SGP was monitored by loss of TCA precipitable counts. After 18 hr of incubation the entire digest was precipitated with 1ml of 10% TCA. The precipitate was filtered on nitrocellulose, washed and measured for ^{14}C radioactivity.

4.6.2 BETA-AMYLASE DIGESTION

Fifty microliters (50ul) containing 4907cpm of prelabelled muscle SGP was incubated with 100mM sodium acetate pH 5.0 buffer and 0.5mM dithiothreitol with approximately 2U of beta-amylase in a 100ul reaction mixture at room temperature. Hydrolysis of glucose was monitored in exactly the same manner as described before.

4.6.3 GLUCOAMYLASE DIGESTION

Fifty microliters (50ul) containing 4907cpm of prelabelled muscle SGP was incubated with 100mM sodium acetate pH 5.0 buffer and 4U of glucoamylase in a 100ul reaction mixture at room temperature. Hydrolysis of glucose was monitored in exactly the same manner as described before.

4.7 Digestion of Prelabelled SGP with alpha-and beta-Glucosidase

Prelabelled SGP was prepared from 500ul of a16 fold purified sample of the enzyme in a 1000ul reaction mixture and used for the following digestions.

4.7.1 ALPHA-GLUCOSIDASE DIGESTION

Three hundred microliters (300ul) containing 25400cpm of prelabelled SGP was incubated with 100mM sodium phosphate pH 7.0 buffer and 5U of alpha-glucosidase at room temperature. At 18 hr, 48 hr and 72 hr time intervals, 100ul aliquots of the digest were taken and precipitated with 1ml 10% TCA. The precipitate was filtered on nitrocellulose, washed and counted for ^{14}C radioactivity.

4.7.2 BETA-GLUCOSIDASE DIGESTION

Three hundred microliters (300ul) containing 25400cpm of prelabelled SGP was incubated with 100mM sodium acetate pH 5.0 buffer and 5U of beta-glucosidase at room temperature. One hundred microliter (100ul) aliquots were taken from the digest at 18 hr, 48 hr and 72 hr time intervals and precipitated with 1ml 10% TCA. The precipitate was filtered on nitrocellulose, washed and counted for ^{14}C radioactivity.

4.8 Digestion of Radioglucosylated Tryptic Peptide from SGP with alpha- and beta-glucosidase

4.8.1 TRYPSINOLYSIS OF PARTIALLY PURIFIED PRELABELLED SGP

A radiolabelled tryptic peptide was prepared from prelabelled SGP, obtained with a 16 fold purified sample of the enzyme in the following manner. Three hundred microliters (300ul) containing 25400cpm of the prelabelled protein was precipitated with an equal volume of 20% TCA and collected by centrifugation. After washing the pellet twice with ether, it was dissolved in 50ul of 8M urea containing 0.4M ammonium bicarbonate at pH 8.0. The sample was heated at 55°C for 15 min

and then diluted to 200ul with water. TPCK-trypsin
(Pierce) was then added to the sample to give a 1 :
25 protease : protein ratio and incubated at 37°C
for 24 hr. Following this, the same amount of
trypsin was added again and the sample incubated
for a second 24 hr period.

4.8.2 RECOVERY OF RADIOGLUCOSYLATED TRYPTIC PEPTIDE

After incubation was completed, the tryptic
digest was applied to a Biogel P-4 column (80 x
0.8cm) equilibrated with 50mM ammonium bicarbonate
buffer and eluted with the same buffer. Fractions
of 0.5ml were collected and 50ul aliquots taken for
measurement of ^{14}C-radioactivity.

4.8.3 ENZYME HYDROLYSIS OF RADIOGLUCOSYLATED
TRYPTIC PEPTIDE

Samples of the radioglucosylated tryptic
peptide (5350cpm each) were digested with alpha-and
beta-glucosidase separately, and each digest
fractionated on the same P-4 column. Fractions of
the same size as in (b) were collected and measured
for ^{14}C-radioactivity.

4.9 Beta-elimination of prelabelled SGP

Two procedures for beta-elimination of
carbohydrate from serine/threonine linked proteins
were performed on prelabelled SGP from sweet corn.

4.9.1 PROCEDURE 1

In this procedure, 210ul containing 15321cpm
of prelabelled SGP from sweet corn (prepared from
16 fold purified enzyme) was incubated with 0.2M
sodium hydroxide and 0.3M sodium borohydride in a
300ul digest at 45°C. Beta-elimination of glucose
was determined by measuring any loss of TCA
precipitable counts. At 15 hr and 42 hr time
intervals 150ul aliquots were taken and
precipitated with 1ml of 20% TCA. The precipitate

was filtered on nitrocellulose, washed with 10% TCA and water and the filter paper measured for ^{14}C-radioactivity. As a control, 155ul containing 15213cpm of prelabelled SGP from rabbit heart (prepared from 20 fold purified enzyme) was incubated with 0.2M sodium hydroxide and 0.3M sodium borohydride in a 300ul digest at 45°C. Beta elimination of glucose was determined in exactly the same manner.

4.9.2 PROCEDURE 2

A sample containing 200ul of prelabelled SGP (14591cpm) from sweet corn, prepared from 16 fold purified enzyme was precipitated with an equal volume of 20% TCA. The pellet was collected by centrifugation and washed twice with 1.5ml of ether. The pellet was redissolved in 0.5ml of 0.5M NaOH and a 250ul aliquot taken and precipitated with 1ml of 20% TCA. The precipitate was filtered on nitrocellulose, washed with 10% TCA and water and the filter paper measured for ^{14}C-radioactivity. The rest of the solution was incubated at 4°C for 24 hr. After incubation, the digest was measured for TCA-precipitable counts in exactly the same manner to determine whether any beta-elimination of glucose occurred.

5.   ENZYMOLOGY OF SWEET CORN SGP

5.1   pH optima of sweet corn SGP

Most enzymes function within a limited pH range and exhibit maximal activity at a specific hydrogen ion concentration. A few operate in a broad range of pH. The sweet corn SGP not only functions over a wide range of hydrogen ion concentrations but exhibits maximal activity peaks

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at two separate pH values 4.5 and 8.5. In other
words, the enzyme has a double pH optima.
Furthermore, the enzyme is about 3 times more
active at pH 8.5 than at pH 4.5.

This uncommon pH-activity curve for the sweet
corn SGP, especially the difference of 4 units
between the two maximal activity peaks, suggested
that perhaps two separate activities were being
measured. The measurements were performed with
partly pure enzyme that was 80 fold purified. The
sample, however, were free from any lipid-
glucosylating activity and when radioglucosylated,
only the 42kDa SGP is radiolabelled. When the
sample was fractionated on Sepharose Cl-6B there
was an enrichment of the pH 8.5 activity in the
early fractions of the SGP peak suggesting that,
perhaps there were two separate but similar SGP's.
However, analysis of the glucosylated protein after
self glucosylation at pH 4.5 and 8.5 revealed that
the products, at least, were similar in that both
were stable to alkali and the added glucose were
removable by beta-glucosidase. When both products
were analyzed by SDS-PAGE each had the same
molecular weight. It appears, therefore, that
there is only one SGP that has an uncommon double-
pH optima. The result of the enrichment of the pH
8.5 activity in the early fractions of Cl-6B
remains unresolved.

5.2 Inhibition of sweet corn SGP
     When a cell-free extract is assayed, an almost
negligible amount of glucosylating activity is
detected. The glucosylating activity is only
expressed after the extract is dialyzed. Apparently
a low-molecular weight inhibitor(s) is present in

the extract that inhibits both the self-
glucosylating protein and the lipid-glucosylating
activity. Furthermore, the inhibitor is stable to
heat as a heated extract inhibits the glucosylating
activity in a dialyzed extract.

The endogenous inhibitor in the sweet corn
extract was not identified. However, ATP was found
to be a potent inhibitor to the sweet corn SGP.
Rabbit muscle SGP is inhibited by ATP with 50%
inhibition observed at a concentration of 3.5mM.
ATP is a 30-fold more potent inhibitor of sweet
corn SGP with 50% inhibition occuring at a
concentration of 100uM.

The self-glucosylating protein in rabbit
muscle is inhibited by p-nitrophenyl-saccharides,
namely p-nitrophenylglucose, p-nitro-
phenylmaltose, p-nitrophenylmaltotriose and p-
nitrophenyl- maltotetrose. These synthetic
compounds inhibit by acting as alternative
substrates. The rabbit muscle SGP functions by
transferring glucose from UDPglucose to a pre-
existing oligosaccharide chain attached to itself
via tyrosine, extending the chain to eight glucose
units long. These p-nitrophenylsaccharides, by
mimicking the acceptor site, can act as acceptors
for the transferred glucose. In addition, this
behaviour of the p-nitrophenylsaccharides further
demonstrates the function of rabbit muscle SGP in
synthesizing alpha-1,4 glucosidic bonds. p-
Nitrophenol, p-nitrophenyl-alpha-glucoside and p-
nitrophenyl-beta-glucoside were tested on sweet
corn SGP and no effect on its activity was
observed. This observation indicated that there
was a fundamental difference in the self-

glucosylating activity between the muscle and sweet
corn proteins. In addition arginine and a synthetic
peptide comprising the same amino acid sequence as
the glucosylated tryptic peptide T2 were examined
for any inhibitory effect on the activity of the
sweet corn SGP. No effect was observed, indicating
that the enzyme is unable to utilize these as
substrates.

5.3 Dependence of sweet corn SGP on divalent cations

The muscle SGP is stimulated by manganese
ions. Sweet corn SGP is completely dependent on
divalent cations for its activity. When treated
with EDTA the sweet corn SGP looses all its
activity. The concentration dependence of sweet
corn SGP on various divalent cations was measured.
Manganese has the most potent stimulating effect at
a concentration of 5mM. Magnesium also activated
SGP but to a lesser extent than manganese,
achieving a maximum stimulating effect of about 50%
at 10mM concentration. Calcium had no stimulatory
effect on SGP activity.

5.4 Radioglucosylation in the absence of $MnCl_2$

When a cell-free extract is assayed for SGP in
the absence of Mn^{2+} ion, in addition to the SGP, a
number of proteins of higher molecular weight
became radiolabelled. Of these, the most prominent
radiolabelled band occurs in the region of
approximately 70kDa. This form of apparent
radioglucosylation occurs within the 30 min
incubation period allowed for the SGP assay. The
activity, however, was present only sometimes and
in most preparations was absent.

A similar form of radioglucosylation has been detected with partially purified extracts (16 fold) in which proteins of molecular weight higher than SGP become radiolabelled in the absence of manganese ions. However, this form of radioglucosylation is detectable only after prolonged incubation and occurs over a 24 hr period. In addition the reaction proceeds in the presence of 1mM $MnCl_2$ but is inhibited by 5mM of the divalent cation. Furthermore, UDPglucose as well as ADPglucose serve as substrates for the reaction. This is unlike SGP which utilizes UDPglucose as a substrate but not ADPglucose.

The radiolabelled proteins formed during this 24 hr incubation period range in molecular weight between 42kDa and 200kDa and appear as sharp distinct bands on a radioautograph following SDS-electrophoresis. Evenly spaced radiolabelled bands appear between 42kDa and 70kDa separated by approximately 3kDa.

The formation of these higher molecular weight products could arise from elongation of the carbohydrate component of SGP. In this case, the radiolabelled bands would represent SGP with different amounts of carbohydrate. In order to test this hypothesis, the radiolabelled products were incubated with glycogenolytic enzymes in an attempt to remove the radiolabel from the proteins. It was found that the radiolabel was resistant to hydrolysis by alpha-amylase, glucoamylase, phosphorylase and isoamylase. Consequently the radiolabelled products were incubated with cellulase to determine whether the radioactive glucose was attached in a beta-linkage. Cellulase

failed to remove the radiolabel. It appears therefore that these higher molecular weight products are not starch-like or cellulose-like molecules and represents some other form of glucosylation. There is no evidence that these proteins are related to SGP.

6. PRIMING ABILITY OF SWEET CORN AMYLOGENIN (SGP)

6.1 Experimental strategy

If the sweet corn SGP was the protein primer upon which the starch molecule was constructed, then it may be possible to synthesize starch in vitro by incubating the primer with the starch synthesizing enzymes, and the glucose donor substrates (UDPglucose and ADPglucose). The synthesis of starch on the protein primer could be detected by monitoring the increase in the molecular weight of the protein as the carbohydrate increases in size. This could be accomplished by SDS-PAGE which can demonstrate any change in the molecular weight of the protein.

This approach was used to determine whether sweet corn SGP could act as a primer for the synthesis of polysaccharide. The SGP was prelabelled by allowing the protein to undergo self-glucosylation with UDP-[^{14}C]glucose. This permitted the detection of the protein by radioautography. Cell-free extracts of sweet corn were used as a source for the starch synthesizing enzymes. It was possible that, in addition to starch synthase and branching enzyme, other enzymes were involved in the synthesis of starch. If SGP was a primer, a 'chain extender' enzyme may be

necessary to extend the single glucose into an
oligosaccharide before starch synthase and
branching enzyme can act.

6.2 Sweet corn SGP is a primer

The prelabelled protein, therefore, was
incubated with cell-free extracts of corn and
millimolar concentrations of UDPglucose and/or
ADPglucose. At different time intervals aliquots of
the incubation mixture were analyzed by SDS-PAGE
and radioautography. After 1 hr of incubation, the
appearance of a radiolabelled band at the top of
the gel occurred and this was also observed for 2
hr and 3 hr time periods. In addition, there was a
decrease in the amount of radiolabel at 42kDa, the
position of prelabelled SGP. The decrease in
intensity of the SGP band was moreso at 2 hr and 3
hr.

The appearance of the radiolabelled band at
the top of the gel could only originate from the
prelabelled SGP. It appears, therefore, that the
SGP was converted into a large molecular weight
molecule by incubation with both UDPglucose and
ADPglucose suggesting that the protein can function
as a primer in polysaccharide synthesis.

6.3 Demonstration of the Priming Ability of Sweet
Corn SGP

The priming ability of sweet corn SGP was
examined by molecular-weight-shift experiments
carried out in 3 stages.

6.3.1 PREPARATION OF PRELABELLED SGP

Prelabelled SGP for this purpose was prepared
from a 16 fold purified extract by incubating a
sample of the enzyme with 2uM UDP-[^{14}C]glucose, 5mM

$MnCl_2$ and 50mM Tris-HCl pH 7.4 buffer at room temperature for 20 min. Following incubation, the reaction mixture was applied to a Sephadex G-25 column (6 x 1.5cm) equilibrated with 50mM Tris-HCl pH 7.4 buffer containing 2mM CHAPS and eluted with the same buffer. Fractions (1ml) were collected and the prelabelled SGP was detected by measuring 50ul aliquots for ^{14}C-radioactivity.

6.3.2 INCUBATION OF PRELABELLED SGP WITH 1MM UDPG AND/OR ADPG

The prelabelled SGP, (typically about 10000cpm) was incubated with 1mM UDPglucose or 1mM ADPglucose or both and an equal volume of dialyzed or undialyzed sweet corn extract at room temperature in 1ml digests. At 1 hr, 2 hr, 3 hr and 24 hr time intervals 250ul aliquots were taken and frozen to stop the reaction. The protein was precipitated with an equal volume of 20% TCA and collected by centrifugation. The pellets were washed twice with ether and dried.

6.3.3 ANALYSIS BY SDS-PAGE AND FLUOROGRAPHY

The dried pellets were dessolved in 50ul of solubilizing buffer by incubation for 24 hr followed by strong vortexing. SDS-PAGE were performed on 10ul of the sample followed by fluorography.

7.   EXPERIMENTAL BACKGROUND TO THE INVENTION

In order to examine in detail the relationship between amylogenin and starch synthesis rate and other starch synthetic enzymes, experiments were conducted on maize plants growing under defined temperature regimes. In these experiments the maize endosperm developmentally regulated expression of amylogenin was found to parallel that

of starch deposition, with minimal amylogenin
activity early in endosperm development and
increasing amylogenin expression as starch
deposition progressed until physiological maturity
of the grain.

Results demonstrated that the initiation of
starch synthesis in maize endosperm is attributable
to the co-ordinated expression of several enzymes
involved in the pathway of starch synthesis.
Continued starch deposition is dependent on a
continued expression of amylogenin which acts as an
important control-point in the pathway.

7.1 Developmental aspects of the formation of corn
SGP in relation to starch synthesis

The appearance of SGP during development must
precede or coincide with the onset of starch
production if the protein is a primer for starch
synthesis. The formation of SGP at a later time
will certainly preclude it from any involvement in
the biogenesis of starch. Therefore an examination
of the formation of SGP during development,
particularly the period during which the
polysaccharide is laid down in the cell, would
provide considerable support for or against the
hypothesis that the protein is a precursor of
starch.

An examination of the formation of SGP and
different components of the starch synthesizing
system was performed on the endosperm of B73
commercial dent corn sampled at 6-30 days after
pollination. The enzyme activities of SGP, ADPG
pyrophosphorylase, ATP-dependent fructokinase, ATP-
dependent glucokinase, branching enzyme, bound

starch synthase, ATP-dependent phosphofructokinase,
PPI-dependent phosphofructokinase,
phosphoglucomutase, soluble starch synthase,
sucrose synthase, UDPG pyrophosphorylase and UTP-
dependent fructokinase were measured in terms of
enzyme concentration, as units/gram dry weight and
enzyme content as units/endosperm with the results
expressed as a percentage of the maximum value
attained. The starch content, dry weight of the
endosperm, the number of endosperm cells, number of
starch granules, the albumin content and
concentration and the rates of starch accumulation
were also measured.

It was found that SGP was already present at 6
days after pollination where other enzymes
important in starch synthesis such as sucrose
synthase, bound starch synthase, soluble starch
synthase and branching enzyme are low in activity
at this time. These enzymes reach their peak along
with SGP at 10 days after pollination. The relative
rate of starch accumulation is greatest between 10
and 14 days after pollination.

The appearance of SGP well before the
accumulation of starch and its peak concentration
occurring together with the other starch
synthesizing enzymes is consistent with the protein
being a precursor of starch.

7.2 Formation of SGP and enzymes involved with the
starch synthesizing system during development in
B73 commercial dent corn
     B73 commercial dent corn was harvested at
different times between 6-30 days after pollination
inclusively. The enzyme activities of SGP, ADPG

pyrophosphorylase, ATP-dependent fructokinase, ATP-dependent glucokinase, branching enzyme, bound starch synthase, ATP-dependent phosphofructokinase, PPI-dependent phosphofructokinase, phosphoglucomutase, soluble starch synthase, sucrose synthase, UDPG pyrophosphorylase and UTP-dependent fructokinase were measured in terms of enzyme concentration, as units/endosperm. These were expressed as a percentage of the maximum value measured. In addition, the starch content, dry weight of the endosperm, the number of endosperm cells, number of starch granules, the albumin content and concentration and the rates of starch accumulation were also determined.

7.3   Purification of Prelabelled SGP from B73 Commercial Dent Corn

The isolation of prelabelled self-glucosylating protein from B73 commercial dent corn was achieved in a 800 fold purification over the cell-free extract. The protein was partially purified in an active state by successive precipitations with acid and salt followed by fractionation on DEAE-Sepharose. At this stage the 27 fold purified enzyme was allowed to undergo self-glucosylation and the prelabelled protein purified further.

The prelabelled protein was purified by successive fractionations on Sephadex S300, Mono Q and C_4. The degree of purification and yield at each stage of the protocol is listed in Table 3.

TABLE 3
Purification of sweet corn amylogenin

Purification Step	Specific Activity (cpm/mg protein)	Overall Yield %	Fold Purific'n
crude extract	1750	100	1.0
pH 5.0 supernatant	2900	88	1.7
50% Ammonium Sulphate ppt	9200	82	5.2
DEAE-Sepharose	48300	78	27.6
5300	140000	62	80.0
Mono Q	168000	53	96.0
C4	1400000	31	800.0

The purified protein had a specific activity of 3.5×10^5 cpm/mg protein. Only 25% of the glucose, however, was radiolabelled. Therefore the calculated specific activity of the B73 corn SGP with all the incorporated glucose being radiolabelled would be 1.4×10^6 cpm/mg protein. The specific activity of the radiolabelled glucose was 4.4×10^{14} cpm/mole of glucose. This implied that the B73 dent corn SGP had incorporated 0.13 mole of glucose into each mole of protein.

7.3 Purification of Prelabelled SGP from B73 Commercial Dent Corn

The isolation of self-glucosylating protein from B73 commercial dent corn was carried out with cobs sampled at 18 and 22 days after pollination. The protein was isolated in a prelabelled form and

was achieved in two phases.

7.3.1 PHASE 1

This phase of the purification was carried out in exactly the same manner as described for phase 1 in the purification of prelabelled SGP from sweet corn. The protein was partially purified and allowed to undergo autoglucosylation, 25% with UDP[^{14}C]glucose and the remaining 75% with unlabelled UDPglucose. The prelabelled protein was then purified further.

7.3.2 PHASE 2, PURIFICATION OF THE PRELABELLED SGP

Step 4: Fractionation by gel filtration chromatography

The prelabelling reaction mixture was applied to a Sephadex S-300 column (2 x 60cm) equilibrated with 50mM Tris-HCl pH 7.4 buffer containing 0.02% triton and eluted with the same buffer. Fractions of 3ml each were collected and the prelabelled SGP detected by measuring 50ul aliquots from alternating fractions for ^{14}C-radioactivity. The fractions containing the prelabelled SGP were analyzed by SDS-PAGE and those that showed the highest protein purity were combined. The pooled fractions were concentrated by centrifugation using centriprep-10 concentrators.

Step 5: Fractionation by fast protein liquid chromatograpy (FPLC) on Mono Q

The concentrated sample was submitted to fast protein liquid chromatography (FPLC) on a Mono Q column. Protein was eluted with a linear gradient of NaCl, from 0-0.4M, formed from 50mM Tris-HCl pH 7.4, 0.02% triton (solution A) and 50mM Tris-HCl pH 7.4, 0.02% triton, 1.0M NaCl (solution B). The gradient was applied over a period of 40 min at a flow rate of 1ml/min. Fractions of 1ml were collected. The prelabelled SGP was detected by

measuring the fractions for ^{14}C-radioactivity and
its purity examined by SDS-PAGE. The fractions
containing the best purity were combined and
concentrated.

Step 6: Fractionation by Reverse-Phase HPLC on
C_4

The prelabelled SGP was purified next by
reverse-phase HPLC using a Vydac C_4 column (8 x
40mm). The sample was fractionated in portions of
0.16mg each. When a larger portion containing
approximately 0.32mg was applied to the column a
reduction in the resolution of the SGP containing
peak was observed. Protein was eluted with a
linear gradient of acetonitrile generated by using
0.1% TFA (solution A) and 0.09% TFA containing 84%
acetonitrile (solution B) at a flow rate of
1ml/min. The gradient of 0-100% solution B was
applied over a period of 50 min. Protein was
detected by monitoring the absorbance of the
effluent at 215nm and 295nm simultaneously. Peaks
containing protein were collected manually. The
prelabelled SGP emerged from the column in a single
peak.

7.4   Comparison of the properties of sweet corn SGP (amylogenin) and muscle SGP (glycogenin)

	sweet corn SGP	muscle SGP
mol. wt. (kDa)	42	39
tendency to aggregate	+	+
autocatalytic enzyme	+	+
cross reacts with Ab to glycogenin	+	+
Km for UDPglucose	similar	similar
stimulated by Mn2+	+	+
inhibited by maltose, adenine nucleotides, p-nitrophenyl saccharides	− + −	+ + +
size of carbohydrate moiety after self-glucosylation	1 glc	8 glc
configuration of glycosidic linkage	beta	alpha
amino acid involved in the glucosidic linkage	arginine	tyrosine

8.   GENERATION OF ANTIBODIES TO THE ENZYME

Polyclonal antibodies to purified amylogenin were generated in goats by the procedure of Lomako et al (1988, FASEB J, 2:3097-3103). Sufficient protein to enable the immunisation was obtained by pooling the enzyme subunit isolated as described above from a number of separate experiments. Antisera were prepared against the 39 kD amylogenin

polypeptide.   The antibodies were then tested for
specificity to the amylogenin polypeptides.

The immune sera obtained precipitated
amylogenin activity from crude extracts of maize
endosperm.   Following incubation of extracts with
immune serum and centrifugation of the
enzyme-Igγ-protein A-Sepharose conjugates, the
amylogenin activity was detectable in the pellet
fractions when the washed enzyme-Igγ-protein
A-Sepharose conjugates were assayed directly for
enzyme activity.

In control experiments, pre-immune sera
collected from animals prior to their primary
immunisation with amylogenin did not partition
amylogenin activity.

Western blot analysis of total maize_endosperm
soluble protein using anti-maize endosperm
amylogenin serum revealed the presence of a single
39 kD immunoreactive polypeptide.

Two further types of polyclonal antibody have
been made.   One was generated against rabbit-muscle
glycogenin obtained from rabbit-muscle glycogen.
The other was generated from three synthetic
peptides coupled to bovine serum albumin using
glutaraldehyde.   The peptides were:
GDAMVTDIEAADE
REGANFVRGYPFSLR
NLDFLEMWRPFFQP

9.   IDENTIFICATION OF AN AMYLOGENIN cDNA CLONE FROM MAIZE ENDOSPERM

The antibodies produced above may be used to screen a maize endosperm cDNA library for clones derived from the mRNAs for amylogenin in an _in vitro_ transcription/ translation system.

The cDNA library may also be screened with oligonucleotide sequences derived from the amino acid sequence of amylogenin. Numerous oligonucleotide probes have been synthesised.

An amplified maize endosperm cDNA library has been constructed. Double-stranded cDNA was prepared from oligo dT-cellulose-purified maize endosperm RNA by a method employing RNaseH and _E coli_ DNA polymerase for the synthesis of the second strand, without prior purification of single-stranded cDNA (Gubler and Hoffman, 1983). The antibody and oligonucleotide probes are used to screen this library to identify clones containing cDNA encoding maize endosperm amylogenin.

Clones producing strong positive signals are plaque-purified during additional rounds of screening. DNA is prepared from the putative maize endosperm amylogenin cDNA clones. Following restriction endonuclease digestion of the DNAs with EcoRl and agarose gel electrophoresis, the sizes of the cDNA inserts of these clones are determined. The cDNA insert is labelled with ^{32}P by nick-translation and used to probe a northern blot of poly(A)-containing RNA from maize endosperm. The size of the mRNA bands to which this probe hybridises indicates the size of the maize endosperm amylogenin mRNA. This should be in close

agreement with the estimates from western blots of
maize endosperm amylogenin.

9.1 Molecular cloning of the amylogenin cDNA

An amplified cDNA library (10^6 plaque-forming
units/ml) from corn endosperm constructed on lambda
ZAPII was used as the DNA template for PCR. A
sense primer CC(T/C/A/G)TTCTTCTT(C/T)AA(C/T)AC was
designed from the partial amino acid sequence of
the protein while the antisense primer,
TCGACGGTATCGATAAGC, was from lambda ZAPII
nucleotide sequence. After 30 cycles (95°C for 1
min; 45°C for 2 min and 72°C for 2 min followed by
a final extension step for 6 min at 72°C), the PCR
product was cloned into a PCR 1000 vector
(invitrogen) and sequenced using Sequenase Version
2.0 (USB). A probe about 500 base pairs in length
was obtained containing the deduced sequence for
three peptides already sequenced directly.
Digoxigenin was then incorporated into the cloned
fragment as digoxigenin-11-dUPT by means of PCR,
using the modified nucleotide in a 1:2 ratio with
dTTP. This served as a probe for screening the
cDNA library. Approximately 10^6 plaque-forming
units of lambda ZAPII phage were used to infect E
coil strain XL1-Blue (Stratagene) and after plating
the plaques were transferred to nylon membranes in
duplicate. The hybridisation buffer contained
5xSSC (chloride-citrate), 0.5% blocking reagent
(Boehringer), 0.1% N-laurylsarcosine, 0.02% SDS and
15 ng/ml of DNA probe. After overnight
hybridisation at 65°C, the membranes were washed
with 0.5xSSC and 0.1% SDS at 65°C. Putative
positive clones were detected by the Genius kit
(Boehringer), subjected to three additional rounds
of purification and excised from lambda ZAPII to

yield recombinant pBluescript phagemid carrying
ampicillin resistance, using ExAssist helper phage
in the in vivo excision protocol provided by
Stratagene, which utilises the E coli SOLR strain.

10.   SEQUENCE ANALYSIS OF THE AMYLOGENIN cDNA
CLONES

       The cDNA insert from each clone may be
sequenced using standard techniques.

       Each clone will encode a polypeptide
equivalent to a partial or a complete amylogenin
protein. The hydropathy index (Kyte and Doolittle,
1982) and secondary structure predictions (Chou and
Fasman, 1978) may be calculated for the encoded
protein using the programmes of Devereux et al
(1987). The Kyte and Doolittle (1982) hydropathy
profile indicates the hydrophobic and hydrophilic
domains within the protein and should be consistent
with its location in the cell (Kaiser and Bassham,
1979). The secondary structure predictions of Chou
and Fasman (1978) may be used in comparisons of the
maize endosperm and animal glycogenin and/or
amylogenin proteins.

       The various amylogenin and/or glycogenin cDNA
sequences may be compared to indicate the extent of
homology. A detailed comparison of the nucleotide
sequences of the cDNA will identify base
substitutions, insertions or deletions, and hence
the amino acid changes produced in the derived
protein sequences.

       One amylogenin cDNA clone has been sequenced

by the dideoxy method using Sequenase Version 2.0
DNA Sequencing Kit (USB). The cDNA clone encoding
amylogenin was deposited at the American Type
Culture Collection, 12301 Parklawn Drive,
Rockville, Maryland 20852, USA under the terms of
the Budapest Treaty on 19 August 1993 under the
accession number ATCC 69389.

Partial cDNA sequences corresponding to
amylogenin from B73 maize are given below in Tables
4 and 5. These match-up with the amino acid
sequences of some of the peptide fragments shown
above.

TABLE 4

SEQUENCE (I)

TGAACTTGGCCTTTGACCGTGAGCTCATTGGTCCGGCTATGTACTTCGGTC
TCCTGGGTGATGGTCAGCCTATTGGTCGCTACGACGATATGTGGGCTGGGT
GGTGTGTCAAGGTGATCTGTGATCATTTGGGATTGGGAGTGAAGACGGGTC
TTCCCTACATCTACCACAGCAAGGCGAGCAACCCATTTGTGAACCTGAAGA
AGGAGTACAAGGGAATTTTCTGGCAGGAGGACATCATGCCTTTCTTCCAGA
GTGCAAAGCTCTCGAAAGAAGCTGTGACGGTTCAACAATGCTACATTGAGC
TGTCCAAGATGGTGAAGGAGAAGCTTAGCGCCATTGATCCTTACTTTGACA
AGCTTGCTGATGCTATGGTGACTTGGATTGACGCTTGGGATGTGCTTAACC
CGGCCACATAAG

TABLE 5

SEQUENCE (II)

CTTCCGTTCTTCTTTAACACCTTGTACGATCCCTACCGTGAAGGTGCTGAC
TTCGTCCGTGGATACCCTTTCAGTCTCCGTGAGGGTGTTTCCACTGCTGTT
TCTCACGGTCTCGGGCTCAACATCCCTGATTACGACGCCCCAACTCAACTC
GTCAAGCCTAAGGAAAGAAACACAAGGTATGTGGATGCTGTCATGACCATC
CCAAAGGAACACCTTTGGCCAATTGTGTGGCATGAACTGCC

The cDNA clones may also be compared by restriction mapping. Restriction maps of the maize endosperm amylogenin cDNAs are constructed to demonstrate their relatedness.

Sequences may also be compared to other glycogenin and/or amylogenin polypeptides. For example, the amino acid sequences derived from the maize amylogenin cDNAs may be aligned with the amino acid sequences derived from an animal glycogenin cDNA (Cohen et al, 1990). The homologies between amino acid sequences are subsequently calculated on the basis of this alignment.

11.   IDENTIFICATION OF A MAIZE ENDOSPERM AMYLOGENIN gDNA CLONE

The cDNAs derived as above may be used to probe a maize genomic library to isolate maize amylogenin genomic DNAs.

In addition, the oligonucleotide probes generated from the amylogenin N-terminal amino acid sequence information may be used to screen the maize genomic library.

12.   CONSTRUCTION OF TRANSFORMATION CASSETTE

The glycogenin and/or amylogenin gene transformation construct requires the presence of an amyloplast transit peptide to ensure its correct localisation in the amyloplast. It is believed that chloroplast transit peptides have similar sequences but other potential sources are available such as that attached to ADPG pyrophosphorylase

(Plant Mol Biol Reporter, 1991, 9:104-126). Other
potential transit peptides are those of small
subunit RUBISCO, acetolactate synthase,
glyceraldehyde-3P-hydrogenase and nitrite
reductase. For example,
 consensus sequence of the transit peptide of small
subunit RUBISCO from many genotypes has the
sequence:
MASSMLSSAAV--ATRTNPAQAS MVAPFTGLKSAAFPVSRK
QNLDITSIA SNGGRVQC;
 the corn small subunit RUBISCO has the sequence:
MAPTVMMASSAT-ATRTNPAQAS AVAPFQGLKSTASLPVARR
SSRSLGNVA SNGGRIRC;
 the transit peptide of leaf starch synthase from
corn has the sequence:
MA ALATSQLVAT RAGLGVPDAS TFRRGAAQGL RGARASAAAD
TLSMRTASARA APRHQQQARR GGRFPSLVVC;
 the transit peptide of leaf glyceraldehyde-3P-
dehydrogenase from corn has the sequence:
MAQILAPS TQWQMRITKT SPCATPITSK MWSSLVMKQT
KKVAHSAKFR VMAVNSENGT;
 the putative transit peptide from ADPG
pyrophosphorylase from wheat has the sequence:
RASPPSESRA PLRAPQRSAT RQHQARQGPR RMC.

    It is possible to express the glycogenin
and/or amylogenin enzyme constitutively using one
of the well-known constitutive promoters such as
CaMV35S but there may be biochemical penalties in
the plant resulting from increased starch
deposition throughout the entire plant. Deposition
in the endosperm is preferred. Possible promoters
for use in the invention include the promoters of
the starch synthase gene, ADPG pyrophosphorylase
gene, and the sucrose synthase gene.

                    SUBSTITUTE SHEET

13. INSERTION OF GLYCOGENIN AND/OR AMYLOGENIN DNA
INTO MAIZE

The glycogenin and/or amylogenin DNA may be
transformed into either protoplasts or other
tissues of a maize inbred line or population. The
existing gene promoters in genomic DNA ensure that
the extra genes are expressed only in the
developing endosperm at the correct developmental
time. The protein sequences likewise ensure that
the enzymes are inserted into the amyloplast.

14. TRANSGENIC PLANTS AND PROGENY

Transgenic maize plants are regenerated and
the endosperms of these plants are tested for
increased glycogenin and/or amylogenin enzyme
activity. The kernels are also tested for enhanced
rate of starch synthesis at different temperatures,
starch yield, etc.

The plants are then included in a breeding
program to produce new maize hybrids with altered
starch synthesis.

CLAIMS

1. A method of producing a plant with altered starch synthesising ability comprising stably incorporating into the genome of a recipient plant at least one donor gene encoding a starch primer.

2. A method as claimed in claim 1 in which the plant has an increased capacity to produce starch.

3. A method as claimed in claim 1 or claim 2 in which the plant has an ability to synthesise starch with an altered structure.

4. A method as claimed in claim 1 in which at least one of the donor genes encodes amylogenin.

5. A method as claimed in claim 1 in which at least one of the donor genes encodes glycogenin.

6. A method as claimed in claim 1 in which at least one of the donor genes encodes protoglycogenin.

7. A method as claimed in claim 1 in which at least one of the donor genes is in the sense orientation and encodes all or part of the starch primer.

8. A method as claimed in claim 1 in which at least one of the donor genes is in the antisense orientation and encodes all or part of the starch primer.

9. A method as claimed in claim 1 in which at least one of the donor genes encodes a modified allelic form of the enzyme.

10.   A method as claimed in claim 1 in which at least one of the donor genes is derived from a plant.

11.   A method as claimed in claim 1 in which the recipient plant is of the family <u>Gramineae</u>.

12.   A method as claimed in claim 11 in which the recipient plant is of the species <u>Zea mays</u>.

13.   A method as claimed in claim 1 in which the recipient plant is a tomato.

14.   A plant and progeny thereof having at least one donor gene encoding a starch primer stably incorporated into the plant's genome such that the plant's ability to synthesise starch is altered.

15.   A plant as claimed in claim 14 in which at least one of the donor genes encodes amylogenin.

16.   A plant as claimed in claim 14 in which at least one of the donor genes encodes glycogenin.

17.   A plant as claimed in claim 14 in which at least one of the donor genes encodes protoglycogenin.

18.   A plant as claimed in claim 14 which is of the family <u>Gramineae</u>.

19.   A plant as claimed in claim 18 which is of the species <u>Zea mays</u>.

20.   A plant as claimed in claim 14 which is a tomato.

21.   Seed of a plant as claimed in claim 14.

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22.   A plant genome which is homozygous for the donor gene or
      genes encoding the said starch primer.

23.   A hybrid plant of which at least one parent is a plant as
      claimed in claim 22.

24.   A DNA construct comprising a DNA sequence encoding
      amylogenin.

25.   A DNA construct comprising a DNA sequence encoding
      glycogenin.

26.   A DNA construct comprising a DNA sequence encoding
      protoglycogenin.

27.   A DNA construct comprising a DNA having the nucleotide
      sequence shown in Table 4.

28.   A DNA construct comprising a DNA having the nucleotide
      sequence shown in Table 5.

29.   A DNA construct comprising all or part of a cDNA sequence
      as deposited at the American Type Culture Collection on
      19 August 1993 under the accession number ATCC 69389.

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FIG.1

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FIG. 2

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FIG. 3

Enzymes of starch synthesis

Sucrose

Sucrose Synthase

Fructose   UPD glucose

Hexokinase   UDP Gppase

F-6-P ⟷ G-6-P ⟷ G-1-P

Isomerase   Mutase

F1,6bP

G-1-P

ADP Gppase

Triose-P

ADP glucose

SSSynthase
B.Enzyme   BSS

SGP ⟶ SGP-glucan

Amylogenin   Amylogenin   Amylose

Amylopectin

Starch granule

Seed

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FIG.4

UDPG +

Glycogen
initiator
synthase

Protein
primer

Protein primer
containing
oligosaccharide
(1, 4 - α - glucan)
chains

Glycogen synthase

+ Branching enzyme
+ UDPG

Glycogen molecules
built onto the
protein backbone

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FIG.5

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FIG.6

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FIG.7

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FIG.8

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FIG. 9

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(54) Title: NOVEL PLANTS AND PROCESSES FOR OBTAINING THEM

(57) Abstract

Plants with an altered starch synthesising ability are produced by incorporating into the genome of the plant at least one donor gene encoding a starch primer. The starch primer is an enzyme capable of inititiating starch synthesis, such as amylogenin and/or glycogenin and/or protoglycogenin. DNA constructs encoding a starch primer are provided.

Enzymes of starch synthesis

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   C12N15/82   C12N15/54   A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   C12N   A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	FASEB JOURNAL. 1992 FASEB MEETING, ANAHEIM, CALIFORNIA, APRIL 5-9. 1992. vol. 6, no. 4 , 26 February 1992 , BETHESDA, MD US page A1520 GIEOWAR-SINGH, D., ET AL. 'Purification of self-glucosylating protein from sweet corn'	22,23
Y	see abstract 3382 --- -/--	24,27-29

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 1994	28 -02- 1994

| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016 | Authorized officer Maddox, A |

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page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	see abstract 6829 ---	24,27-29
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A	WO,A,92 11382 (CALGENE) 9 July 1992 see example 1a ---	1-29
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1

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